Interleukin-4 Antagonizes Neutrophil Functions during Acute Bacterial Infection

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Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

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Zürich, 2016

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I SUMMARY

Neutrophils belong to the innate immune system and are usually the first cell type recruited from the bone marrow and periphery to a site of inflammation. Neutrophils exert multiple effector functions to modulate inflammation and fight invading pathogens. Defects in their generation, recruitment or effector functions can lead to severe disease states. It has been shown that patients suffering from T helper 2 cell (Th2)-mediated diseases are more prone to bacterial infections, suggesting a negative effect of Th2-type immune responses on anti-bacterial immunity. We hypothesized that interleukin-4 (IL-4), a central cytokine driving and maintaining Th2 immunity, could affect neutrophils and lead to increased susceptibility towards infections.

Acute bacterial infection led to expansion and migration from the bone marrow (BM) to blood and spleen of $CD11b^+$ $Ly6G^+$ neutrophils, which was dependent on granulocyte colony-stimulating factor (G-CSF). Similarly, injection of long-acting G-CSF, in the form of G-CSF/anti-G-CSF antibody complexes (G-CSFcx), caused expansion and migration of neutrophils. Significantly, IL-4, in the form of long-acting IL-4/anti-IL-4 antibody complexes (IL-4cx), antagonized the effects of G-CSF both following bacterial infection and G-CSFcx administration. This inhibitory effect of IL-4cx was mediated by direct binding of IL-4 to neutrophils, in particular to the type II IL-4 receptor (IL-4R) present on neutrophils. G-CSFcx enhanced type II IL-4R expression on neutrophils, thereby making them even more responsive towards IL-4. Mice treated with G-CSFcx were able to survive systemic Listeria monocytogenes infection, whereas co-administration of IL-4cx led to mortality rates comparable to untreated mice. Furthermore IL-4R deficiency protected from lethal Listeria monocytogenes infection. IL-4cx decreased neutrophil egress from BM by CXCR4 upregulation on neutrophils, as well as decreased CXCR2 expression in splenic neutrophils. Moreover, IL-4 signals inhibited, via the p38 MAPK pathway, the migration in vitro and in vivo of neutrophils towards CXCR2-binding chemokines. Notably, the migration capacity of neutrophils into skin lesions infected with group A Streptococcus was dampened in animals receiving IL-4cx, leading to more severe disease; in contrast, neutralizing

endogenously produced IL-4 led to smaller skin lesions and more rapid bacterial clearance in mice. IL-4 signals also diminished the production of reactive oxygen species by neutrophils.

These data show a previously unknown role of the IL-4–type II IL-4R pathway in affecting multiple functions of neutrophils and thereby impacting anti-bacterial immunity.

II ZUSAMMENFASSUNG

Neutrophile Granulozyten (kurz: Neutrophile) gehören zu dem angeborenen Immunsystem und sind normalerweise die ersten Zellen, die, aus dem Knochenmark und der Peripherie, von einem Entzündungsherd rekrutiert werden. Neutrophile verfügen über verschiedenste Effektorfunktionen um sowohl Entzündungen zu kontrollieren, als auch eindringende Pathogene zu bekämpfen. Defekte in der Entstehung, der Migration oder in der Funktionalität von Neutrophilen können demzufolge zu schweren Krankheitsbildern führen. Es wurde gezeigt, dass Patienten, welche an T-Helferzellen 2 (Th2 Zellen) vermittelten Krankheiten leiden, vermehrt zu bakteriellen Infektionen neigen, was zu der Annahme führt, dass eine Th2 Immunantwort einen negativen Einfluss auf das Immunsystem bezüglich bakteriellen Infektionen hat.

Unsere Hypothese war, dass Interleukin-4 (IL-4), ein zentrales Zytokin in der Entstehung und Erhaltung einer Th2 Immunantwort, einen Einfluss auf Neutrophile haben könnte und dadurch die Anfälligkeit der Patienten für Infektionen erhöht wird. Wir konnten zeigen, dass eine akute bakterielle Infektion zu einem erhöhten Level des Granulozyten-Kolonie-stimulierenden Faktors (G-CSF) im Blut führte, was wiederum zu einer vermehrten Expansion von $CD11b^+$ $Ly6G^+$ Neutrophilen im Knochenmark und einem erhöhten Austritt dieser Zellen aus dem Knochenmark in das Blut und die Milz führte. Injektionen von lang wirksamen G-CSF, in der Form von G-CSF / anti-G-CSF Antikörper Komplexen (G-CSFcx), führte zu einer vergleichbaren Expansion und Migration von Neutrophilen. IL-4, appliziert in der Form eines lang wirksamen IL-4 / anti-IL-4 Antikörper Komplexes (IL-4cx), führte zu einer signifikanten Antagonisierung des G-CSF vermittelten Effektes, sowohl nach einer bakteriellen Infektion als auch nach systemischer Gabe von G-CSFcx. Dieser inhibitorische Effekt von IL-4cx wurde durch die direkte Bindung von IL-4 an den IL-4 Rezeptor (IL-4R), spezifischer an den von Neutrophilen exprimierten Typ II IL-4R, vermittelt. G-CSFcx führte zudem zu einer erhöhten Expression des Typ II IL-4R in Neutrophilen, wodurch die Sensitivität gegenüber IL-4 zusätzlich erhöht wurde. Mäuse, die mit G-CSFcx vorbehandelt wurden, überlebten eine systemische Infektion mit Listeria monozytogenes, wohingegen die zusätzliche Gabe von IL-4cx zu einer ähnlichen Mortalitätsrate wie bei unbehandelten Tieren führte. Weiter konnten wir zeigen, dass Mäuse, die nicht über den IL-4R

verfügten, eine für Wildtyp-Tiere ansonsten letale systemische Listeria Monozytogenes Infektion überlebten. IL-4cx führte zu einem verminderten Austritt von Neutrophilen aus dem Knochenmark, sowohl durch deren vermehrte Expression von CXCR4 im Knochenmark als auch durch Herabregulation von CXCR2 in der Peripherie. Zusätzlich inhibiert IL-4, vermittelt durch den p38 MAPK Signaltransduktionsweg, die Migration in vitro und in vivo gegenüber CXCR2-bindenden Chemokinen. Bemerkenswert ist, dass die Migrationsrate von Neutrophilen in eine mit Gruppe A Streptokokkus infizierte Hautläsion in Tieren, die mit IL-4cx behandelt wurden, vermindert war, was zu einem schwereren Krankheitsbild führte. Neutralisierung von endogen produzierten IL-4 hingegen, führte im Vergleich zu unbehandelten Mäusen, zu kleineren Hautläsionen und einer schnelleren Clearance der Bakterien. Zusätzlich führte IL-4cx auch zu einer verminderten Produktion von reaktiven Sauerstoffspezies.

Diese Daten zeigen eine bis anhin unbekannte Rolle des Type II IL-4R Signalwegs in der Beeinflussung von verschiedenen Funktionen der Neutrophilen, wodurch die antibakterielle Immunantwort beeinträchtigt wird.

III INTRODUCTION

The immune system can be divided into the innate and adaptive parts of the immune system. This thesis focuses on a subset of granulocytes, the neutrophils, which are a key player of the innate immune system. An innate immune response involves also various other cell types, including other subsets of granulocytes including eosinophils and basophils, monocytes, macrophages, natural killer (NK) cells and innate lymphoid cells. These cells all express certain pattern recognition receptors (PRR) that are not changed by recombination during the cell's development. Another common feature of these cells is that they are activated within minutes of an infection, and they are able to fight a broad range of pathogens efficiently [1].

Eosinophils

Eosinophils, a subset of granulocytes, are produced in the bone marrow from hematopoietic stem cells (HSC), and are under baseline conditions mostly found in the gastrointestinal tract where they normally reside within the lamina propria. Classically these cells are known to defend their host against parasitic helminths as they increase in numbers and release their cytotoxic granule by piecemeal degranulation in the environment of a parasite. Upon activation by cytokines, in particular interleukin-5 (IL-5), immunoglobulins and complement eosinophils start to secrete a variety of pro-inflammatory cytokines and chemokines, thereby influencing the surrounding tissues and cells [2, 3]. Eosinophils can act furthermore as antigen-presenting cells (APC) as they can process and present various microbial and parasitic antigens [4].

Basophils

Basophils represent the smallest subset of granulocytes. Similar to neutrophils and eosinophils, they as well arise in the bone marrow, where they fully mature and are released into the peripheral blood. In steady state they are mainly found in the periphery, whereas they migrate into tissues during pathologies such as parasitic infections or allergy. Basophils are a major source of IL-4 and can additionally even function as APCs, thereby inducing differentiation of naïve T cells into T helper 2 (Th2) cells [5].

Monocytes

Monocytes, belonging to the group of mononuclear phagocytes, completely depend on colony-stimulating factor 1 (also known as M-CSF) for their development and survival in mice. Monocytes arise from myeloid precursor cells in the bone marrow [6].

Monocytes expressing high levels of lymphocyte antigen 6C ($LY6C^{hi}$) in mice represent 'classical monocytes' that are recruited rapidly to sites of inflammation and can act as precursors of peripheral mononuclear phagocytes by differentiating into macrophages and dendritic cells (DC). The rapid recruitment of monocytes is a key component of the host response to pathogenic insult [6]. Monocytes however do not appear to contribute to the maintenance of peripheral tissue macrophages in the steady state which was shown in parabiotic mice [7].

Once inside a tissue, monocyte-derived macrophages respond to microenvironmental cues that determine whether they contribute to the establishment of the local inflammatory response or to its resolution. These opposing activities probably depend on sequential recruitment waves of $LY6C^{hi}$ monocytes that acquire different activities in response to the dynamic changes that occur in the tissue environment. In a model of experimental autoimmune encephalomyelitis (EAE) mice deficient in CCR2 or treated with a CCR2-depleting antibody were protected from developing EAE, indicating monocytes or monocyte-derived macrophages as disease promoters in this disease model [8]. However, it should be noted that in other diseases, monocyte-derived macrophages can also have beneficial effects, for example during the recovery from spinal cord injury [9].

Macrophages

Macrophages also belong, as the above-mentioned monocytes, to the mononuclear phagocytic system. Depending on their tissue localization und functional phenotype macrophages are divided into specialized subpopulations, for example the Kupffer cells in the liver and the osteoclasts in the bone. They mediate critical functions in remodeling of tissues, both during development and in the adult animal.

Classically activated macrophages (also termed M1 macrophages) defend the host from a variety of pathogens and have roles in antitumor immunity. The other macrophage

subpopulations display an immunosuppressive and anti-inflammatory phenotype and include the alternatively-activated macrophages (also named M2 macrophages), which are important in wound healing. Other non-classical macrophage subsets are the 'regulatory' macrophages which can secrete large amounts IL-10, the tumor-associated macrophages (TAMs), and the myeloid-derived suppressor cells which might be the precursors of TAMs [10, 11].

Macrophages sense signals, which are associated with invading pathogens and dying or dead cells, through PRRs including Toll-like receptors (TLR) or C-type lectin receptors. Therefore macrophages are constantly surveying their surrounding tissues and phagocytose with high efficiency invading organisms. Phagocytosed microorganisms get trapped in a phagosome that then fuses with a lysosome, in which enzymes and toxic free radicals digest and destroy the ingested microbe. The first-responder macrophages usually show an inflammatory phenotype and secrete pro-inflammatory mediators such as tumor necrosis factor (TNF), nitric oxide (NO) and IL-1, which all participate in the activation of various antimicrobial mechanisms.

Tissue stress, including acute and chronic infection, further drives the production of monocytes in a process that is dependent on cytokines, such as granulocyte colony-stimulating factor (G-CSF), and chemokines. This increased production is found in many different types of stress and leads to so-called 'emergency myelopoiesis', during which monocytes quickly differentiate into macrophages and DCs at the site of danger [11].

1 Neutrophils

1.1 Neutrophils during steady-state conditions

1.1.1 Origin and development of neutrophils

Neutrophils belong to the hematopoietic system and therefore arise from HSCs in the bone marrow. Among these, short-term reconstituting HSCs show a more limited self-renewal potential and derive from long-term reconstituting HSCs. Short-term reconstituting HSCs give rise to multipotent progenitors (MPPs), which in turn generate lineage-committed oligopotent progenitors, including common lymphoid progenitors (CLP) and the common myeloid

progenitors (CMP). The latter can further differentiate into granulocyte-monocyte progenitors (GMP). This developmental cell type gives rise to the different subpopulations of granulocytes to which also neutrophils belong [12-15] (**Figure 1**).

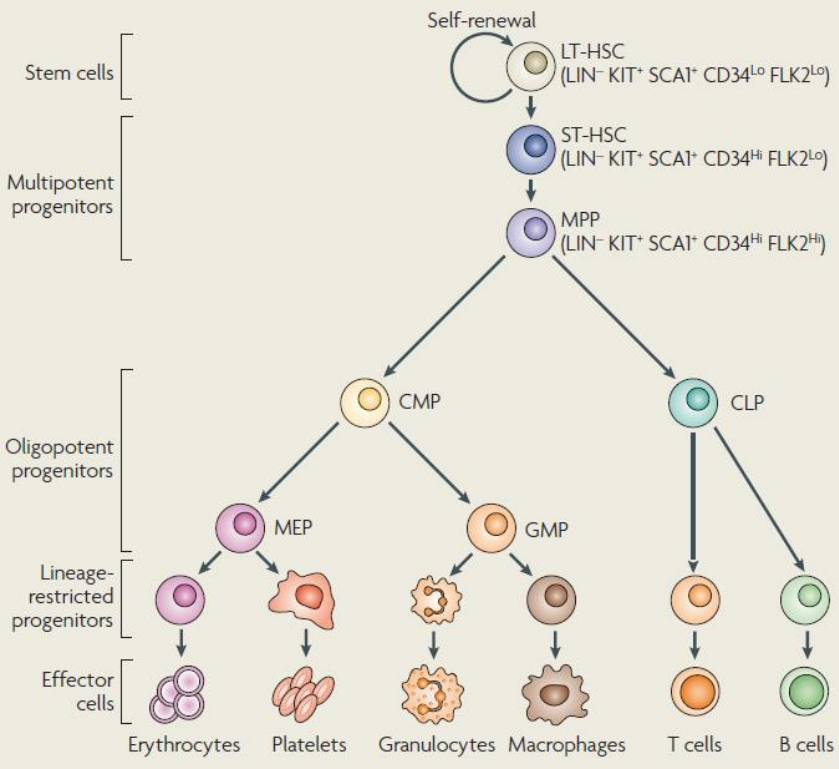

Figure 1. Hematopoiesis. All hematopoietic cells are generated from a small population of hematopoietic stem cells (HSC), which comprises long-term reconstituting HSC and short-term reconstituting HSC (ST-HSCs). Although ST-HSCs maintain multipotency, they exhibit more limited self-renewal potential and differentiate further into multipotent progenitors (MPP) and then into lineage-committed oligopotent progenitors including the common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). The precursors of megakaryocyte-erythrocyte progenitors (MEP) and granulocyte-monocyte progenitors (GMP) give rise to erythrocytes or granulocytes, respectively. Figure adapted from [14].

Mouse neutrophils, the preferred model for in vivo studies, differ in important aspects from their human equivalents. This is perhaps best exemplified by the differences in the respective antimicrobial repertoires and the numbers of neutrophils in circulation.

1.1.2 Neutrophil homeostasis and characteristics

In healthy individuals neutrophils are continuously generated in the bone marrow from the aforementioned myeloid precursors. This process is clearly depended on G-CSF as G-CSF-deficient mice show up to 80% reduction in their peripheral blood neutrophil compartment, which can be corrected with administration of recombinant G-CSF [16]. G-CSF has an effect on the commitment of hematopoietic progenitor cells to the common myeloid lineage as it was shown that granulopoiesis is nearly completely dependent on G-CSF receptor signals [17]. Furthermore G-CSF controls proliferation of granulocytic precursors, the release of mature cells from the bone marrow and reduces the transit time through the granulocytic compartment [18].

For a first line of defense against pathogens, a pool of neutrophils is maintained as well in the periphery, namely in blood, spleen, liver and lung. Interestingly, there is a dramatic difference between mice and humans in their pool of circulating blood neutrophils. Whereas human blood is rich in neutrophils, with 50–70% of circulating leukocytes belonging to this subpopulation, circulating neutrophils in mice only make up for 10–25% of blood leukocytes [19]. The average circulatory lifespan of neutrophils is up to 12.5 hours for mouse cells [13].

Their clearance from the circulation occurs mainly in the bone marrow, liver and spleen. Increased CXC-chemokine receptor 4 (CXCR4) expression is seen in aged neutrophils, and this is thought to help direct them back to the bone marrow where they are then eliminated. In the liver neutrophils can be taken up and removed by resident Kupffer cells [20].

Neutrophil homeostasis in peripheral blood is tightly regulated by the following mechanisms:

- Proliferation and differentiation rate of neutrophil precursors in the bone marrow,
- egress of mature neutrophils from the bone marrow into the periphery, and
- neutrophil clearance by the phagocytic system in the spleen, liver and bone marrow [21].

Mature neutrophils have an average diameter of 7–10 µm, their nucleus is segmented and the cytoplasm is enriched with granules and secretory vesicles [13]. They are characterized in humans and mice by the expression of CD11b and, additionally, in mice, by granulocyte differentiation antigen 1 (Gr-1). As Gr-1$^+$ cells include not only neutrophils but also monocytes and macrophages, the lymphocyte antigen 6 complex – locus G (Ly6G), which together with Ly6C is a component of Gr-1, is used to distinguish neutrophils from other myeloid cells [22, 23].

1.1.3 Emergency granulopoiesis

In a state of severe, systemic bacterial infection the pool of present neutrophils is rapidly used up and, to ensure an adequate protection, rapid de novo generation of neutrophils has to occur. This state is called emergency granulopoiesis, which is characterized by the generation of new cells, as well as by an increased release of immature and mature cells from the bone marrow into the periphery [24].

This process can be divided into three phases. Firstly, the pathogen has to be sensed, resulting in an activation of the immune and hematopoietic systems. Such activation can be mediated indirectly through hematopoietic cells - most likely monocytes and tissue-resident macrophages - and stromal cells. Importantly these cells need to have access to the pathogen and are able upon detection of invading microorganisms through PRRs to alter their gene expression. The release of cytokines, such as G-CSF and granulocyte–macrophage colony-stimulating factor (GM-CSF), Interleukin (IL)-3 and IL-6, stimulate myeloid progenitor cell proliferation and granulocytic differentiation [24]. Also hematopoietic stem and progenitor cells, such as myeloid progenitors, can react directly to invading pathogens through PRRs [25].

In a second step the signals have to be translated. As mentioned above, G-CSF has an important function already in steady state, and it was as shown by several studies that serum G-CSF levels are augmented during an infection [16, 26, 27]. Mice deficient in G-CSF mount an inadequate granulopoietic response upon infection with Listeria monocytogenes, thereby increasing the lethality of the infection compared to wild-type mice [28]. G-CSF signaling induces signaling through signal transducer and activator of transcription 3 (STAT3), which was shown to be essential for granulopoiesis by promoting directly the expression of the transcription factor

CCAAT enhancer-binding protein-β (Cebpb) and binding to Myc promotor, thereby inducing gene expression [29]. The transcription factors CCAAT-enhancer-binding protein-α (C/EBPα) and C/EBPβ were shown to be important in steady-state granulopoiesis and emergency granulopoiesis, respectively [30, 31].

Importantly emergency granulopoiesis needs to be reversed after clearing an infection, allowing the return to steady-state conditions. One such regulatory feedback mechanism is mediated by STAT3 signaling. STAT3 activates the suppressor of cytokine signaling 3 (SOCS3), which acts as a negative feedback regulator of cytokine signaling, including G-CSF and GM-CSF [32]. Therefore, STAT3 exerts positive and negative effects on granulopoiesis.

1.2 Neutrophil migration

1.2.1 Neutrophil egress from bone marrow

A neutrophil spends the majority of its life in the bone marrow and its release form the bone marrow is a rapid way to increase the number of circulating neutrophils available for recruitment into tissues in response to infection or inflammation.

The egress from neutrophils from bone marrow into the periphery has been found to be antagonistically regulated by CXC chemokine receptor (CXCR)2 and CXCR4, which are both expressed on neutrophils. CXCR4 mediates a retention signal to neutrophils in the bone marrow, whereas CXCR2 facilitates their egress. The CXCR4 ligand CXCL12 (also known as stromal cell-derived factor (SDF)-1) and the CXCR2 ligands CXCL1 (also known as KC) and CXCL2 (also known as macrophage inflammatory protein-2; MIP-2) are all constitutively expressed in the bone marrow [33]. Osteoblasts are the major source of CXCL12, whereas endothelial cells are the major cellular source of the CXCR2 ligands in the bone marrow [21].

CXCL12 normally dominates over CXCR2 ligands thereby mediating the retention of neutrophils in the bone morrow. However, upon downregulation of CXCR4, the balance is changed in favor of CXCR2 signaling thus mobilizing neutrophils from bone marrow [34]. Neutrophils in the bone marrow appear to lower their surface expression of CXCR4 during their maturation process,

which could be a mechanism to ensure the mobilization of only functionally mature neutrophils while retaining less mature ones [35].

In acute inflammation, mobilization of neutrophils from the bone marrow is orchestrated by G-CSF. G-CSF mobilizes neutrophils not only by upregulation of CXCR2 and downregulation of CXCR4, but also by shifting the balance between CXCL12 and CXCR2 ligands in the bone marrow. G-CSF reduces on one side the absolute number of osteoblasts, the major cellular source of CXCL12, while simultaneously increasing on the other side CXCL1 and CXCL2 expression in endothelial cells of the bone marrow (**Figure 2**) [18, 21, 34, 36].

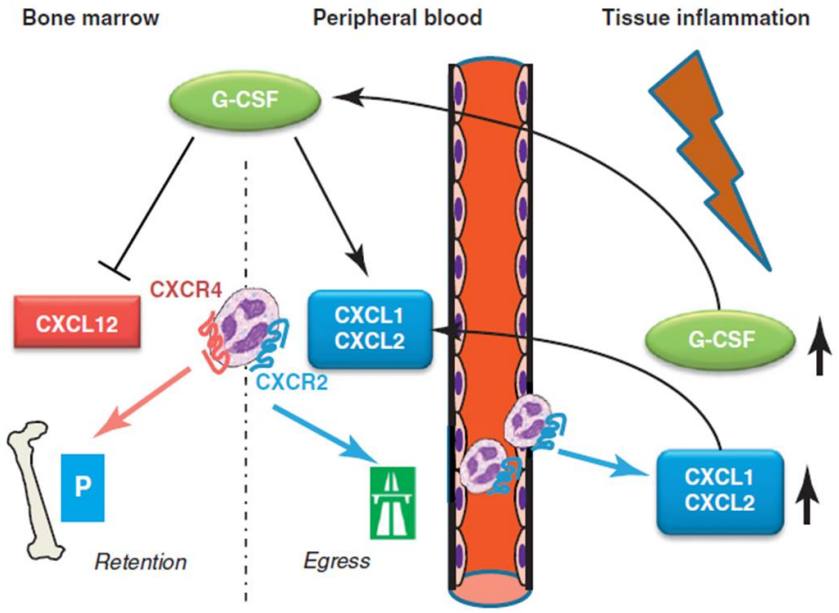

Figure 2. Regulation of neutrophil egress from the bone marrow by CXCR4 and CXCR2. The main effect of the CXCR4 binding ligand CXCL12 (SDF-1) is to retain neutrophils in the bone marrow, whereas the CXCR2 ligands CXCL1 (KC) and CXCL2 (MIP-2) promote neutrophil egress. G-CSF mobilizes neutrophils from the bone marrow by decreasing the ratio of CXCR4 to CXCR2 and altering their ligands' concentration in the bone marrow. Neutrophil egress is influenced by local secretion of G-CSF and CXCR2 ligands within the bone marrow, as well as the production of these mediators from inflamed peripheral tissues. Figure adapted from [21].

Inflammatory mediators released upon inflammation in the affected tissue most likely also act in the bone marrow, thereby contributing as well to the release of neutrophils from the bone marrow. It was shown that intraperitoneal injection of CXCL2 or G-CSF led to increased

peripheral neutrophil blood counts [37]. Thus, chemokines can either act locally to induce neutrophil recruitment into peripheral tissues and at distance to induce neutrophil mobilization from the bone marrow [21].

1.2.2 Extravasation from blood

The extravasation of neutrophils from blood vessels to tissues follows the steps tethering, rolling, adhesion, crawling and transmigration (**Figure 3**).

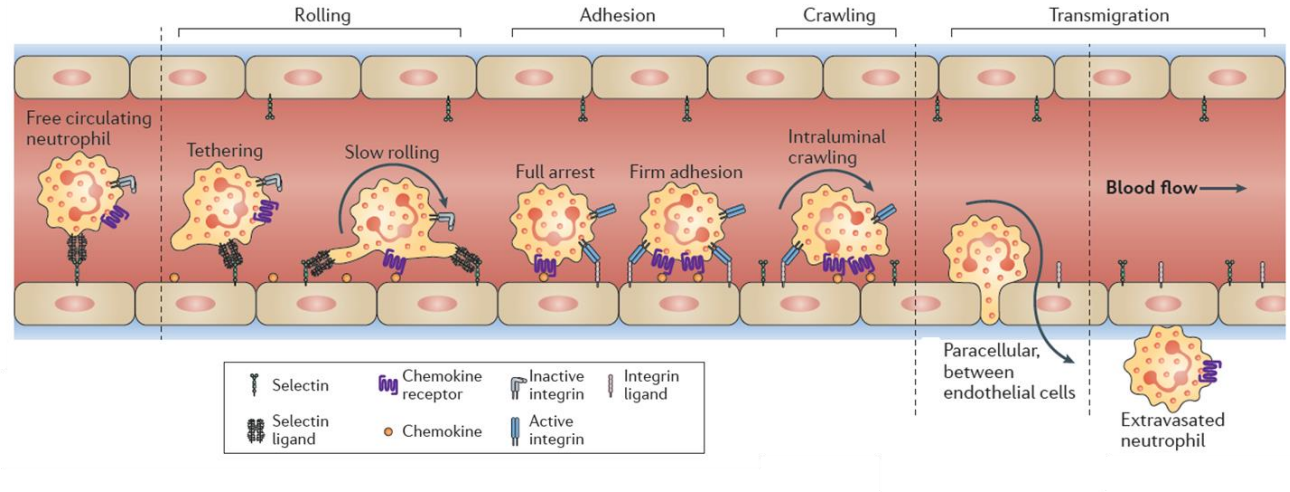

Figure 3. Neutrophil recruitment cascade. The classical pathway of extravasation starts by tethering in a selectin-dependent manner of neutrophils on the endothelium, which goes over to slow rolling and subsequent firm adhesion to the blood vessel wall. In the process of crawling neutrophils follow a chemokine gradient, thereby finding their location to transmigrate the endothelium which occurs paracellular (between endothelial cells) and less frequently also transcellular (through endothelial cells). Figure adapted from [13].

Neutrophils are guided to their site of action through bacterial and host-derived inflammatory signals, such as by histamine or various cytokines, which stimulate nearby endothelial cells to express adhesion molecules, including P-selectin or various integrins, and to synthesize E-selectin [38].

Tethering and the subsequent rolling of neutrophils on the endothelium occur through interaction of selectins with their glycosylated ligands on the cell surface. This process induces activation of neutrophils, which, due to the histotoxic potential of neutrophils, is highly controlled, thereby preventing tissue-specific accumulation and activation in non-inflammatory

states. The first step of neutrophil activation, termed priming, is induced by the interaction of neutrophils with activated endothelial surfaces or by exposure to cytokines (for example TNF-α), chemokines or pathogen-associated molecular patterns (PAMPs) [18]. Maximal neutrophil degranulation and activation of the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase occurs only in cells that have been primed before activation [39].

Chemokines, such as CXCL1 and CXCL2, decorate the activated endothelium and have a crucial but not exclusive role in neutrophil activation. These positively charged molecules are immobilized on endothelium by binding to negatively charged heparan sulphates, which prevents their washing away due to the shear forces [40, 41]. This is crucial as it allows the formation of intravascular chemotactic gradients. The activation of G-protein-coupled chemokine receptors on neutrophils induces a conformational change of the cell surface-expressed integrin's lymphocyte function-associated antigen 1 (LFA1) and macrophage-1 antigen (Mac-1), which then adopt a higher affinity for their ligands intercellular adhesion molecule 1 (ICAM1) and ICAM2. LFA1 binding to ICAM1 is essential for firm adhesion, whereas Mac-1 rather seems to be important for the crawling of neutrophils. Both steps however are crucial to prepare cells for transmigration [42].
Due to shear forces neutrophils start to form so called pseudopods, which allow them to probe their surroundings and to actively crawl in a chemotactic-independent manner to an endothelial cell-cell junction where they can transmigrate into the tissue [13, 43]. Passage across the endothelial cell layer occurs preferentially through paracellular migration (between endothelial cells) but can also be transcellular (through an endothelial cell).

Neutrophils harbor specific groups of proteases, including various metalloproteinase (MMPs) and serine proteases, such as neutrophil elastase, that exert enzymatic activity against extracellular matrix (ECM) molecules. It is unclear yet if neutrophils cut their way through the basement membrane or if proteases help neutrophil emigration through less-porous ECM, as neutrophils preferentially migrated through regions of basement membrane that exhibit low levels of ECM molecules [13, 44].

1.2.3 Neutrophil migration during inflammation and infection

Tissue-resident leukocytes act as sentinels to initiate neutrophil recruitment by controlling and inducing various processes, such as an increase in permeability of local blood vessels and the release of chemokines. These resident cells are alerted by PAMPs and damage-associated molecular patterns that trigger IL-1β, IL-6 and TNF-α secretion through interaction with PRRs, such as Toll-like receptors (TLRs), NOD-like receptors and C-type lectin receptors [45]. Platelets, aggregating with neutrophils via P-selectin, facilitate neutrophil recruitment during acute non-infectious lung injury, including LPS-induced lung inflammation. In addition, platelet-derived neutrophil-attracting chemokine heterodimers of CCL5 and CXCL4 are important for neutrophil recruitment during acute lung injury [13].

Furthermore neutrophils themselves are able to further recruit additional neutrophils to a site of inflammation, as neutrophils produce IL-17, which induces the release of pro-inflammatory factors, such as interferon-γ (IFN-γ), chemokines and MMPs, from mesenchymal and myeloid cells, leading to recruitment and activation of neutrophils [13, 46].

After transmigration through the endothelium neutrophils have to move away from the vessel into the tissue, thereby overcoming the former chemokine gradient. This suggests that there needs to be another chemotactic gradient which overrules the first one. Neutrophils react preferentially to so-called end-target chemoattractants, including bacteria-derived *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and the complement component C5a. These mediators are able to overcome the so-called intermediate signals built by gradients of CXCL1 and CXCL2 [21].

At the molecular level, the activity of the phosphoinositide 3-kinase (PI3K) and p38 mitogen-activated protein kinase (MAPK) pathways is pivotal for the prioritization between opposing signals from end-target and intermediate chemoattractants. PI3K phosphorylates the membrane lipid phosphatidylinositol-3,4-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-triphosphate (PIP3) at the leading edge of the neutrophil. At the same time, the phosphatase and tensin homolog (PTEN) is localized to the back of the crawling cell, thereby converting PIP3 back to PIP2 in areas of the cell that have moved away from the leading edge.

End-target and intermediate chemoattractants both activate PI3K. However, in opposing gradients, PTEN and PIP2 surround the entire cell circumference and no cell migration occurs. But only end-target chemoattractants are able to activate p38 MAPK, which regulates PI3K activity, therefore initiating migration towards the site of inflammation and infection. Accordingly, in neutrophil recruitment to sites of focal necrosis, the CXCL2 signal is hierarchically overridden by formyl-peptide signals as neutrophils leave the zone of maximal CXCL2 concentration (**Figure 4**) [21, 47-49].

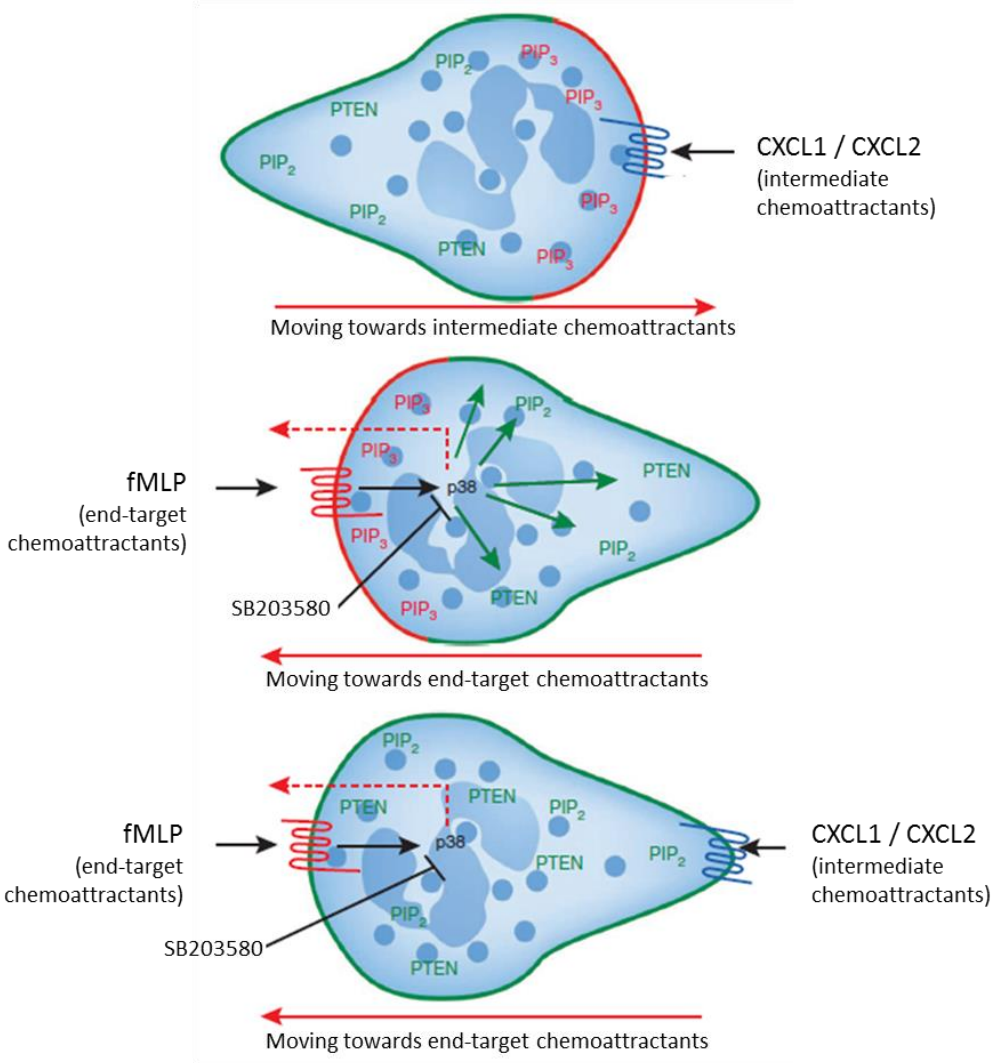

Figure 4. Activity of PI3K and p38 MAPK during neutrophil chemotaxis. Upper panel: Signaling from an intermediate chemoattractant, such as CXCL1 (KC) and CXCL2 (MIP-2), leads to activation of CXCR2 and PI3K thereby increasing PIP_3 (red) and ultimately to cell migration. **Middle panel:** Signaling from an end-target chemoattractant, such as fMLP, activates PI3K and p38 MAPK. This leads to the accumulation of

PIP_3 at the signaling site and subsequent migration towards the signaling site. **Lower panel:** Opposing gradients of end-target and intermediate chemoattractants leads to induction of PTEN in the entire cell, thereby antagonizing PIP_3 accumulation. All chemotaxis occurs then through p38 MAPK, guiding the cell in the direction of the end-target chemoattractant. The small-molecule inhibitor SB203580 is able to inhibit phosphorylation of p38 MAPK, thereby antagonizing its effect. Figure adapted from [42].

Even though it is thought that most neutrophils die in the tissue and are cleared by macrophages, it was also shown that extravasated neutrophils can re-enter the vasculature. A mouse study reported that reverse transmigration occurred owing to a junctional adhesion protein that normally prevents reverse transmigration [50]. In patients with rheumatoid arthritis the presence of a minor population of reverse-transmigrating neutrophils, representing 1–2% of peripheral blood neutrophils, could be detected [51]. The effect of reverse transmigrating neutrophils could be a way of preserving neutrophils that have not participated in fighting the infection. Adversely, neutrophil reverse transmigration could lead to systemic or chronic inflammation as cells re-entering the circulation could disseminate inflammation to other organs [13].

1.3 Neutrophil effector functions

Amulic et al. formulated a simple but elegant statement summarizing the regulation of neutrophil effector functions, which reads as follows: “The basic instruction set of the activated neutrophil is both effective and ruthless in its simplicity: (1) kill microbes, (2) do no harm to the host, and (3) when in doubt, see rule 1.”
Neutrophil effector functions include phagocytosis, the formation of effector granules, respiratory burst, NETosis, and cytokine production.

1.3.1 Phagocytosis

Phagocytosis constitutes a major mechanism to remove pathogens by internalization into a vacuole called the phagosome. This process is active and receptor-mediated, and can occur either directly through PRRs or indirectly via opsonisation. Opsonisation relies either on Fc receptors recognizing IgG antibodies (FcγR) thereby mediating FcγR-mediated phagocytosis or complement receptor-induced phagocytosis. After the pathogen is engulfed the so-far harmless

phagosome undergoes a maturation process to gain its functionality. An important step in this maturation process is the delivery of preformed granules (see 1.3.2) and their antimicrobial content to the phagosome [12, 52].

1.3.2 Neutrophil granules

Characteristic of neutrophils is the presence of different granules. These specialized storage organelles are needed to ensure a safe transport of the dangerous material through the bloodstream and targeted delivery at the appropriate location (**Figure 5**) [12].

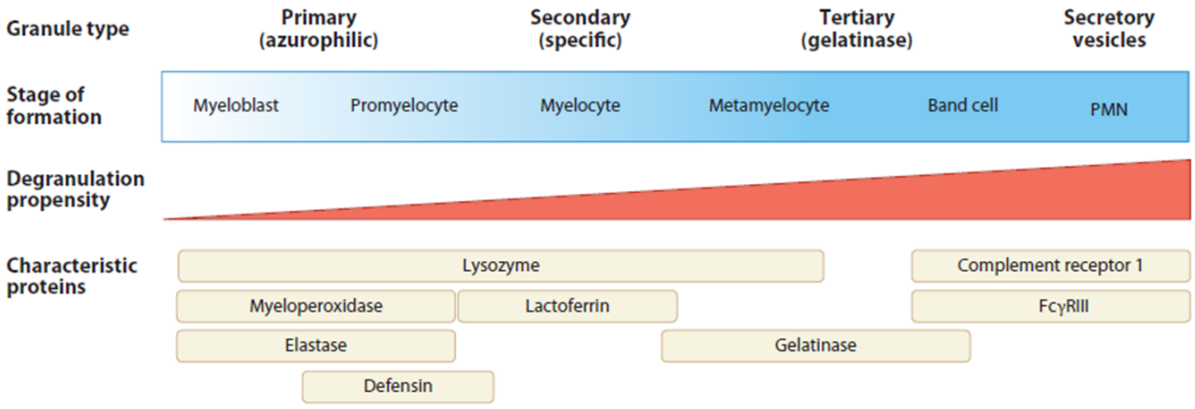

Figure 5. Neutrophil granules. Neutrophil granules are typically divided into three types: primary (azurophilic), secondary (specific), and tertiary (gelatinase). The so-called secretory vesicles are also considered as a subset of granules. Granules differ in their ability to be mobilized within the neutrophil, with secretory vesicles being the first to fuse with the plasma membrane and azurophilic granules demonstrating the least degranulation propensity. Figure adapted from [12].

The largest subsets of granules are the primary (also known as azurophilic) granules, which are the first ones formed during cell maturation. Primary granules contain myeloperoxidase (MPO) among other important proteins, including defensins, lysozyme and various serine proteases.

The two other granule subsets, the secondary (specific) granules and the tertiary (gelatinase) granules, are considerably smaller than primary granules. Secondary and tertiary granules are MPO-negative, but harbor lactoferrin and metalloproteases, respectively [33].

The secretory vesicles are formed through endocytosis in the end-stages of neutrophil maturation and therefore consist predominantly of plasma-derived proteins such as albumin.

The membrane of secretory vesicles is an important reservoir for a number of membrane-bound molecules functioning during neutrophil migration.

Mobilization of different granules follows different kinetics, which associates every subset of granules with a particular stage of neutrophil activation. Secretory vesicles are the first to be mobilized upon binding of neutrophils to the endothelium. Fusion of these granules with the plasma membrane leads to exposure of their content, including fMLP receptors, which is crucial for further neutrophil activation. Further activation signals lead to mobilization of tertiary granules resulting in a release of metalloproteases, which help the neutrophils to overcome the basement membranes. Complete activation at the inflammatory site leads to fusion of the primary and secondary granules with either the plasma membrane, releasing their antimicrobials into the tissue, or with the phagosome, thereby exerting antimicrobial activity. This fusion and the subsequent alteration of the membrane constitution permits assembly of the NADPH oxidase complex and allows the production of reactive oxygen species (ROS), which is called respiratory burst. ROS can be released both inside the phagolysosome and outside the cell, thereby modifying and damaging other molecules [12, 33].

One major content of granules are antimicrobial proteins, which can be classified into three main types [12]:

- cationic peptides, including mostly α-defensins, which might be involved in the inhibition of bacterial cell wall synthesis;
- proteolytic enzymes, including lysozyme destroying bacterial walls, and serine proteases, such as neutrophil elastase;
- proteins that deprive microorganisms of essential nutrients by chelating essential metals from microbes, thereby possibly impacting bacterial growth, such as iron-binding lactoferrin.

1.3.3 Respiratory burst

ROS production occurs in the process of respiratory burst and relies on a variety of different molecules. These varying species all have the potential to modify and damage other molecules and are crucial for neutrophil antimicrobial activity. These processes are mediated by the

NADPH oxidase which is a multiprotein complex that exists in a disassembled state in quiescent cells but is rapidly assembled following phagocyte activation. The reaction occurring is the following (**Figure 6**):

$$NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^- + H^+$$

O_2^- can further react to produce peroxide (H_2O_2):

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

O_2^- and H_2O_2 both show strong cytotoxic effects. Another potent agent is hypochlorous acid (HOCl) which can be formed when MPO, a component of azurophil granules, utilizes H_2O_2 formed during the respiratory burst in the following reaction [12, 53]:

$$H_2O_2 + CL^- + H^+ \rightarrow HOCl + H_2O$$

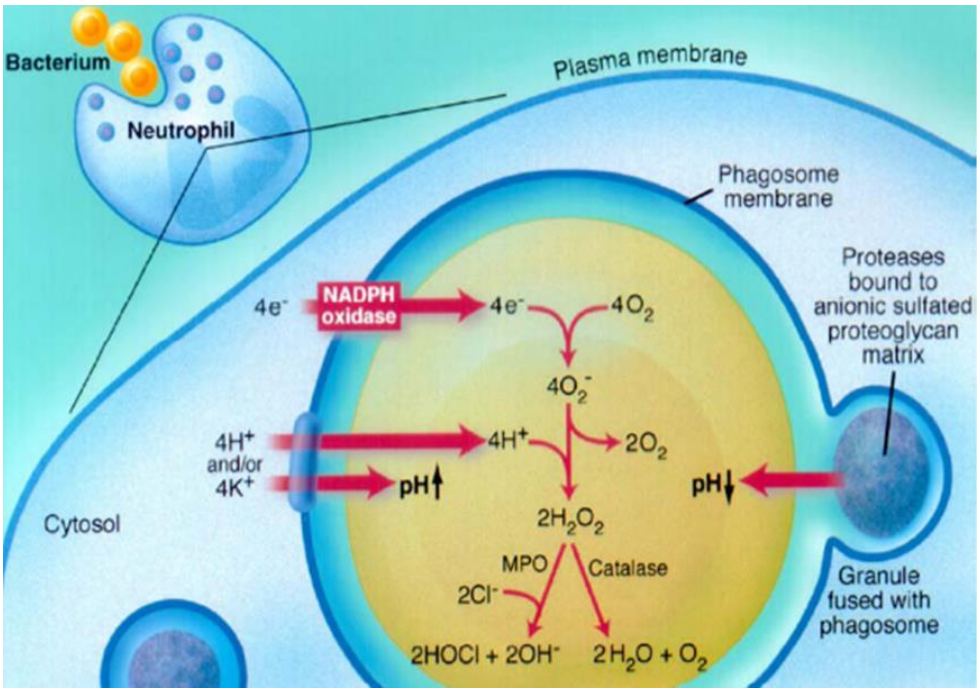

Figure 6. Effects in the phagosome. A number of chemical reactions take place within the phagosome. The newly-assembled NADPH oxidase in the membrane of the phagosome produces electrons (e^-) which reduce molecular oxygen (O_2) to superoxide (O_2^-). Influx of protons (H^+) and other cations compensate

for the negative charge transferred in the compartment. The protons are further used to reduce superoxide to H_2O_2, which can be degraded to oxygen and water in a catalase-dependent reaction. H_2O_2 can as well combine with chloride (Cl^-) to form hypochlorous acid (HOCl) in a reaction catalyzed by myeloperoxidase (MPO). Figure adapted from [54].

Neutrophils need to receive a stimulus to start producing ROS, unstimulated cells do not show any superoxide production. A diverse group of agonists, including physiological stimuli such as phagocytable particles and fMLP are able to induce respiratory burst [53].

1.3.4 NETosis

Upon stimulation, neutrophils can undergo NETosis, an active form of cell death where the cells break down their nucleus content and release so-called neutrophil extracellular traps (NET), which consist of web-like structures of decondensed chromatin, histones and antimicrobial factors, into the extracellular space. NETs were shown to trap many different kinds of microbes; however, it is believed that the release of NETs (also termed NETosis) mainly occurs when neutrophils try to engulf pathogens that are too big to be phagocytosed either because of their large size or because of formation of aggregates [55].

In the process of NET formation, neutrophil elastase is released from azurophilic granules into the cytosol and translocates to the nucleus, where it cleaves histones to decondense chromatin. Also, the ROS pathway, particularly NADPH oxidase and MPO, is involved in NET formation in response to chemical and biological stimuli [12, 55].

1.3.5 Role of neutrophils in disease

The main function of neutrophils is to provide a first line of defense against infections. Therefore altering their generation, migration or effector functions can have various consequences by promoting or downscaling their defensive capacity against pathogens or even by harming the host. Let's have a look at some disease pathologies that are caused by neutrophil dysfunction.

Patients suffering from chronic granulomatous disease (CGD), characterized by an inherited defect of NADPH oxidase complex and thus an inability to produce ROS species, are more

susceptible to infections with extracellular bacteria and fungi, as the neutrophils CGD patients are less efficient in killing microbes indicating the importance of ROS production [12, 56].

WHIM (warts, hypogammaglobulinaemia, infection and myelokathesis) is another genetic immunodeficiency affecting neutrophils. Unlike CGD, neutrophils of WHIM patients are functional; however, WHIM patients are neutropenic, while displaying increased numbers of neutrophils in the bone marrow. This phenotype occurs due a mutation in CXCR4 causing enhanced sensitivity to CXCL12 and therefore promoting retention of neutrophils in the bone marrow [18, 21].

Formation of NETs can contribute to autoimmune disorders as it leads to the exposure of intracellular contents to the extracellular space. In systemic lupus erythematosus (SLE), an autoimmune disease characterized by autoantibodies, the presentation of chromatin, neutrophil components and other nuclear autoantigens in the context of NETs is thought to promote the formation of autoantibodies against these structures [57].

Some pathogens have evolved strategies, such as interference with engulfment, disturbance of phagosome maturation and buffering of the intraphagosomal environment, to overcome attacks by neutrophils or to be able to survive within neutrophils [12].

2 Cytokines influencing neutrophils

Cytokines are small proteins released from various cell types including hematopoietic and stromal cells. Cytokines orchestrate various functions of cells including proliferation, survival, function and apoptosis. Their function can be redundant and can provoke pro- and anti-inflammatory actions. Thus, different cytokines can act synergistically or antagonistically.

2.1 Granulocyte colony-stimulating factor (G-CSF)

G-CSF is known to be essential in the production of neutrophils in health and disease states. For example, G-CSF is mainly responsible for the dramatic increase in neutrophil numbers during infections. Therefore, plasma concentrations of G-CSF are normally low to undetectable, but rise rapidly in response to infection and subsequently decline with recovery [58]. G-CSF signals through its receptor (G-CSFR), which is a homodimeric receptor [59]. Mice lacking G-CSF or G-

CSFR demonstrate chronic severe neutropenia, although a small number of neutrophils are still detected, indicating that other factors might in part compensate for the lack of G-CSF signals [16]. G-CSF is produced upon inflammatory stimulation by endothelial cells, macrophages, epithelial cells and fibroblasts. Release of G-CSF leads to increased production and mobilization of neutrophils within and from the bone marrow. G-CSF can also be produced locally at inflammation sites and influence the functions of neutrophils, for example by inhibiting their apoptosis or increasing their survival. G-CSF primes neutrophils thereby increasing the response of neutrophils to agents such as fMLP [60], however it itself does not directly stimulate phagocytosis or ROS production. This system ensures that only upon inflammatory stimulation the toxic ROS is efficiently produced and that during steady state no harm to other cells is mediated [58].

2.2 Granulocyte-macrophage colony-stimulating factor (GM-CSF)

GM-CSF is mainly known for its stimulatory effects on survival, proliferation and maturation of hematopoietic cells of the myeloid lineage. It binds to the heterodimeric GM-CSF receptor, composed of the binding α chain and signaling β chain. GM-CSF receptors are present on monocytes, macrophages, granulocytes, lymphocytes and endothelial cells. GM-CSF is produced mainly in response to immune stimulation and pro-inflammatory cytokines by various cell types including macrophages, mast cells, T cells, fibroblasts and endothelial cells [61]. In neutrophils, GM-CSF enhances chemotaxis towards fMLP by upregulation of the fMLP receptor. Moreover, GM-CSF itself can also function as a chemoattractant [24, 62].

Not only migration but also certain neutrophil functions are influenced by GM-CSF. Upon stimulation with this cytokine, degranulation, release of ROS and phagocytosis are enhanced [63].

2.3 Tumor necrosis factor-α (TNF-α)

TNF-α can, depending on its concentration, have different effects on neutrophils. At lower concentrations, TNF-α has been shown to inhibit cell death of neutrophils, whereas at high concentrations it has the opposite effect, thereby promoting cell death. Moreover, high levels of TNF-α even reversed the protective effect of GM-CSF on neutrophil viability. This dual role of TNF-α depends most likely on qualitatively different signals emerging from the two TNF-α receptors, TNFR1 and TNFR2. Whereas both TNFRs promote cell death, only TNFR1, showing higher signaling stability than TNFR2, can antagonize neutrophil apoptosis by inducing expression of anti-apoptotic genes [64]. At higher concentrations, TNF-α is able to bind to and signal via both receptors, thereby inducing neutrophil apoptosis via death receptor signaling [65-67]. Furthermore, it was suggested that TNF-α can activate and prime neutrophils for NET formation [12].

2.4 Interleukin-1β (IL-1β)

Neutrophils are not only an important source of IL-1β, but also respond to this cytokine in multiple ways. Moreover, IL-1β exerts indirect effects on neutrophils as its increases the surface expression of endothelial adhesion molecules, thereby facilitating the attachment and extravasation of neutrophils from blood vessels. In cultured endothelial cells it was also shown that IL-1β induced the transendothelial passage of neutrophils. IL-1β further stimulates the production and release of neutrophil chemoattractants by endothelial cells and keratinocytes. Collectively, these effects mediate the influx of neutrophils into the site of tissue damage and inflammation [68, 69]. Injection of IL-1 into patients generally induces neutrophilia, resulting from elevated G-CSF levels in plasma, which in turn promotes neutrophil production, egress from the bone marrow and prolonged survival of these otherwise short-lived cells [70]. However, it was also shown that IL-1β reduced ROS production by neutrophils [69].

2.5 Interleukin-4 (IL-4)

2.5.1 Characteristics and origin of IL-4

IL-4 is a member of the short-chain four-α-helix bundle cytokine family and was discovered in the mid-1980s. Its molecular weight varies between 12 and 20 kDa as a result of variable natural glycosylation. IL-4 shares with IL-13 sequence homology of about 25% on the amino acid sequence level, cell surface receptors, intracellular signaling pathways, and certain functional effects on cells [71, 72]. IL-4 is produced mainly by activated Th2 cells but also by mast cells, basophils, eosinophils and NKT cells. IL-4 is known to induce and maintain the so-called Th2 phenotype of lymphocytes and to recruit and activate IgE-producing B cells, as well as to enhance IgE-mediated immune responses [73, 74]. Th2 immunity will be separately addressed in section I.4. Furthermore, IL-4 was shown to regulate, through IL-4 receptor (IL-4R)-signaling, cell proliferation, apoptosis, and expression of numerous genes, including class II MHC, IgE, or arginase in various cell types, like lymphocytes – especially $CD4^+$ and $CD8^+$ T cells -, macrophages, and fibroblasts, as well as epithelial and endothelial cells [72]. Excessive IL-4 production seems to be suppressed in steady-state regulation at peripheral tissue sites. It was shown that in particular induced regulatory T cells, which dampen type 2 immunity in steady state, are mainly responsible for this negative feedback effect on IL-4 production, even though the exact mechanism is still unclear [75]. In addition IL-4 drives the so-called “alternative macrophage activation” [72].

2.5.2 IL-4 signaling

IL-4 requires for its signaling the IL-4 receptor α (IL-4Rα; also termed CD124), which is able to form a heterodimer with other receptor subunits. To form the type I IL-4R, CD124 assembles with the common gamma chain (γ_c; also known as CD132), which is also a part of the receptors of IL-2, IL-7, IL-9, IL-15 and IL-21. Type I IL-4Rs are only able to signal upon IL-4 binding and no other cytokine has been shown to induce signaling via this receptor; for example, IL-13 is not able to signal through γ_c [76]. Conversely, the type II IL-4R can be activated by IL-4 as well as by IL-13. This receptor consists also of CD124, which forms a heterodimer with IL-13Rα1. Both type

I and type II IL-4Rs signal through the Janus Kinase (Jak) / STAT cascade, with CD124 associating with Jak1, CD132 with Jak3, and IL-13Rα1 with Jak2 (**Figure 7**) [71, 73].

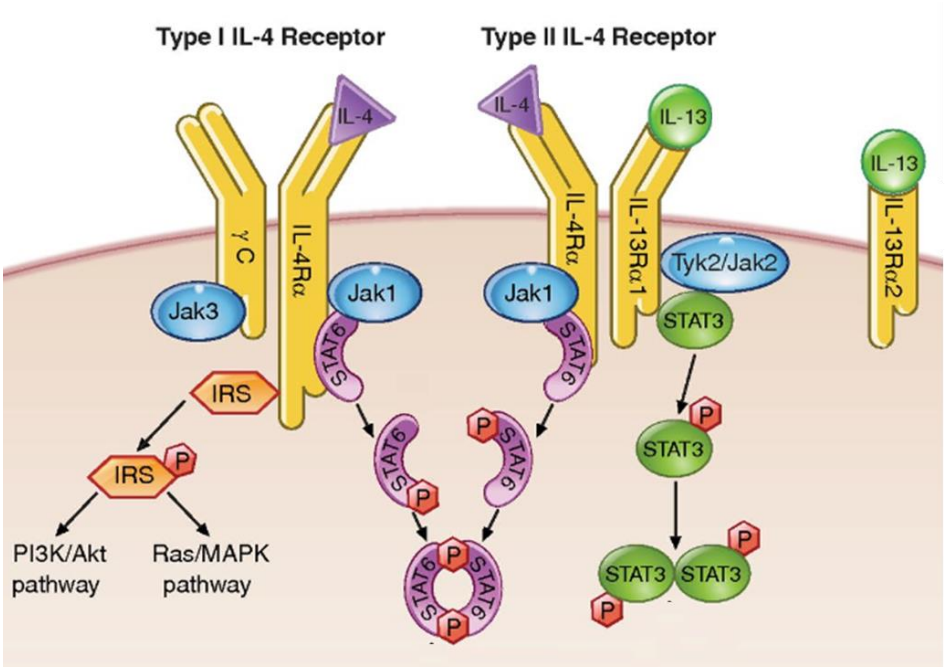

Figure 7. IL-4 signaling pathways. After binding of IL-4 to IL-4 receptor α (IL-4Rα; also termed CD124), heterodimerization of IL-4Rα with either γ_c (CD132) or IL-13Rα1 occurs, thereby forming the type I or type II IL-4R. Dimerization leads to stimulation of Janus kinase (Jak), which then results in subsequent phosphorylation and activation of signaling molecules, such as signal transducer and activator of transcription 6 (STAT6), STAT3, and insulin receptor substrate (IRS). IRS then can initiate other pathways such as the phosphoinositide 3-kinase (PI3K)-Akt and Ras-mitogen-activated protein kinase (MAPK) pathways. Figure adapted from [76].

Triggering of CD124 leads to the activation of STAT6. This was shown by using cell lines, such as Ramos and A549 cells, that express selectively either type I IL-4Rs or type II IL-4Rs, respectively. On the other hand, STAT3 was only induced upon IL-4 binding to cells carrying type II IL-4Rs, thus associating IL-13Rα1 with STAT3 signaling [73].

A second mechanism of signal transduction mediated through IL-4 depends on the insulin receptor substrate (IRS) family. The IRS cascade engages two different pathways, PI3K-Akt, which is mainly involved in cell growth, survival and protein synthesis and Ras-MAPK, being important mainly in cell proliferation and differentiation [76].

As mentioned above, the type II IL-4R is also able to signal upon IL-13 binding. However, the sequence of receptor assembly markedly differs between IL-4 and IL-13. To form either one of the IL-4Rs, IL-4 first assembles with CD124 (Kd = 1 nM), followed by low-affinity interaction with either CD132 (Kd = 559 nM) or IL-13Rα1 (Kd = 487 nM), respectively, resulting in lower overall complex stabilities [73]. Conversely, IL-13 first binds to IL-13Rα1 and then recruits CD124, resulting in the same dimeric receptor used by IL-4 [71] (**Figure 8**). When A549 cells, expressing only type II IL-4Rs, were treated with either IL-4 or IL-13, IL-4 stimulated the phosphorylation of STAT6 at five- to ten-fold lower doses than IL-13. Cells harboring only Type I IL-4R show in terms of STAT6 phosphorylation upon IL-4 stimulation a similar dose response as Type II IL-4R expressing cells do [73].

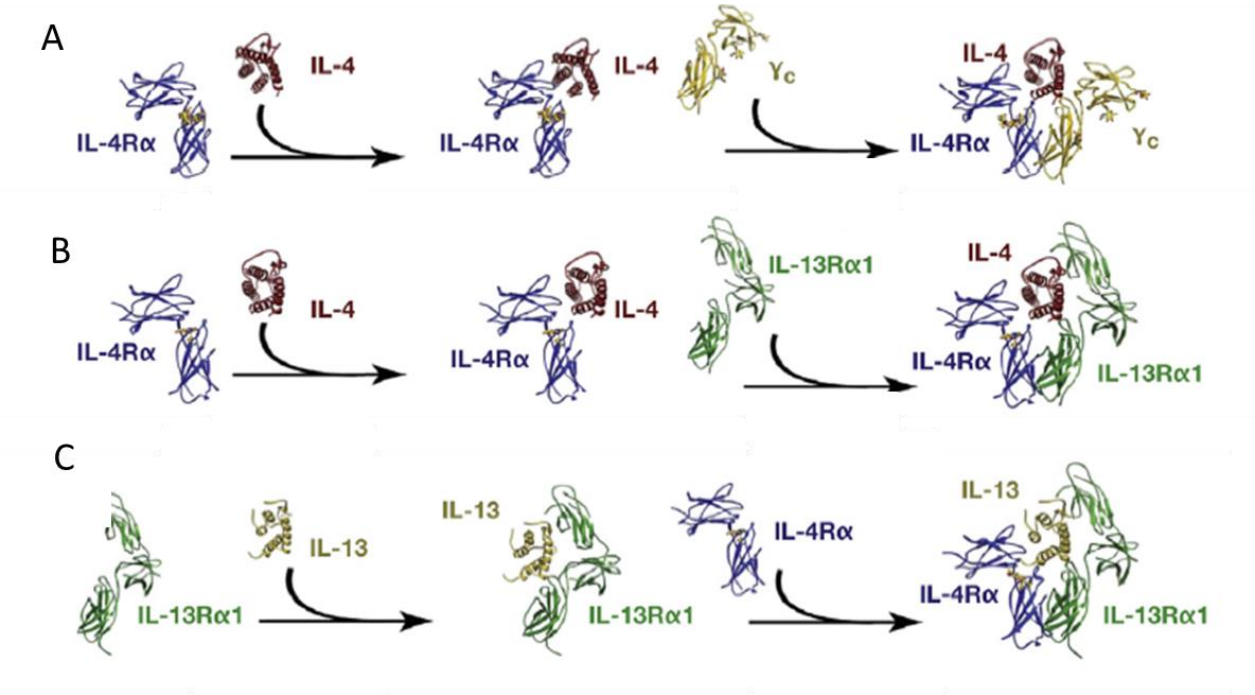

Figure 8. IL-4 receptor assembly. (A and B) Shown is the formation of IL-4Rs where in both IL-4R types IL-4 first binds to IL-4Rα (CD124) and subsequently either (**A**) to γ_c thereby forming the type I IL-4R or (**B**) to IL-13Rα1 thus leading to the assembly of the type II IL-4R. (**C**) IL-13, which can also signal through the type II IL-4R, first binds to IL-13Rα1 and subsequently recruits IL-4Rα. Figure adapted from [73].

2.5.3 IL-4 and neutrophils

A study in human neutrophils suggested that neutrophils only expressed type I IL-4Rs and triggering of these IL-4Rs induced phosphorylation of p38 MAPK, Erk-1/2, Jak1 and Jak2 [77]. However now it is known that Jak2 is mainly associated with IL-13Rα1, being controversial to the statement that no Type II receptor is present [78]. They further found that upon IL-4 signaling SOCS3 mRNA and expression was increased, a critical molecule for negative regulation of cytokine signaling thereby having negative effects on granulopoiesis [32, 77]. Another study showed that IL-4 is able to delay apoptosis in a 24 hours culturing period [79]. In an assay performed using the promyeloblast cell line HL-60 it was shown that IL-4 can shift the balance between neutrophilic and monocytic linage towards neutrophils and drive neutrophil maturation [80].

In a mouse model of inflammatory arthritis it was demonstrated that infiltrating $CD11b^{+}$ myeloid cells, in particular neutrophils, expressed very high levels of CD124 and that administration of IL-4 was able to protect from joint inflammation. The authors further found that CD124 upregulation was a general response to various inflammatory stimuli, such as TLR4 activation by lipopolysaccharide (LPS). However, LPS-induced upregulation of CD124 was at least a two-step process as in mixed bone marrow chimeras of wild-type and $TLR4^{-/-}$ LPS showed CD124 upregulation on both cell types. These results suggested that inflammation leads to the release of soluble factors mediating the increase of CD124 expression, leading most likely to higher sensitivity towards IL-4 [81].

In the airpouch model, which serves as a system for neutrophil migration and accumulation, it was shown that IL-4 injection into the pouch attracts leukocytes, including neutrophils [82]. Others however found that IL-4 is an endogenous inhibitor of neutrophil infiltration [83]. Systemic administration of IL-4 prior to initiating migration into an airpouch by IL-1β led to a decreased influx of neutrophils into the pouch [84].

3 Cytokine complexes

Cytokines are known to have a low molecular weight and upon in vivo administration their half-lives are in the range of minutes. This makes the use of cytokines difficult as cytokines have to be administered either continuously or in very high doses, thereby increasing the risk of cytokine-induced adverse side effects. Several strategies have tried to address these drawbacks of cytokine therapy. The easiest way to increasing the duration of cytokine exposure is simply to inject more cytokine. Another strategy is to prolong the cytokine's half-life for example through PEGylation of the cytokine or by forming cytokine complexes. Cytokine complexes consist of a cytokine and anti-cytokine monoclonal antibody (**Figure 9A**) [85].

Most of the work on cytokine complexes was done with IL-2. Complexing of IL-2 with particular anti-IL-2 antibodies significantly prolonged the half-life of IL-2 and allowed the direction of IL-2 towards specific immune cell subsets [85, 86]. IL-4 complexes were first described by Sato et al. and shortly after in another publication by Finkelman et al. in 1993. They reported that forming complexes by IL-4 and a corresponding neutralizing antibody enhanced the half-life of IL-4 by several fold [87, 88], which we have been able to confirm (**Figure 9B**).

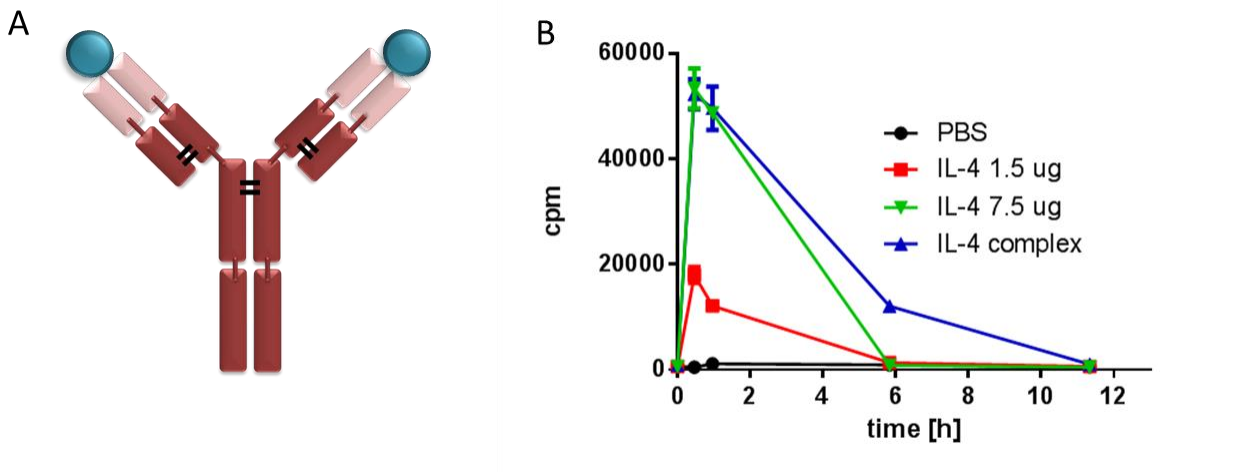

Figure 9. IL-4 cytokine complexes. (**A**) Schematic representation of IL-4 (blue) bound to anti-IL-4 antibody (red). (**B**) Serum bioactivity, as measured by the proliferation of NFS-60 cells ex vivo, at different time-point following one single intravenous injection of low-dose IL-4 (red), high-dose IL-4 (green), or IL-4/anti-IL-4 complexes (IL-4 complex; using low-dose IL-4), given to mice.

4 Type 2 immunity

Infections with parasitic worms, including helminths, or allergic disorders, such as asthma, allergic rhinitis, food allergies and atopic dermatitis, share a common feature in that these disorders provoke a so-called allergic or "type 2" immune response, characterized by the presence of Th2 cells. This immune response can mediate protection against helminths; but type 2 immune responses can also promote acute and chronic inflammatory responses against a diverse range of allergens [89, 90]. In allergic diseases this immune response is induced towards normally harmless environmental antigens and these disorders show an increasing prevalence in the world [91].

Type 2 responses are characterized by the induction of $CD4^+$ Th2 cells, which secrete cytokines known as type 2 cytokines, such as IL-4, IL-5, IL-9, and IL-13. As stated above, IL-4 is able to promote B cell responses and immunoglobulin E (IgE) production, which bind to high-affinity IgE receptors (FceR1) present on basophils and mast cells, leading to their activation and secretion of several cytokines and inflammatory mediators such as histamine and serotonin. However, many other cell types, such as alternatively-activated macrophages and DCs, are also involved in the orchestration of Th2 cell responses. In the field of Th1 and Th17 cell responses considerable developments have been made in understanding their generation and mechanisms, however less is known about how Th2 cell responses are initiated and orchestrated [89, 91].

Apart from Th2 cells, the innate immune system appears to play an important role in the allergic disease atopic dermatitis (AD). Thus, patients suffering from AD show more frequent bacterial skin infections and Th2 immune responses appear to affect protective immunity against persistent Staphylococcus aureus colonization. In psoriasis, a Th1/Th17-driven skin disease, only 6% of the patients suffer from bacterial infection compared to up to 30% in AD patients, suggesting that Th2 cytokines, but not a disturbed skin homeostasis, are responsible for the increased susceptibility towards infections. Already in 1973 it was discovered that in lesional biopsies of AD patients neutrophils were absent, even when the lesion was infected with Staphylococcus aureus. The severity of this neutropenia positively correlated with several

disease markers. Most likely the decreased neutrophil influx was due to a defect in the chemotactic system and additionally impaired effector functions of neutrophils could be detected [92].

IV MATERIALS AND METHODS

1 Animals

C57BL/6 (B6), CD45.1 (Ly5.1)-congenic, CD132$^{-/-}$, Rag1$^{-/-}$, CXCR2$^{-/-}$ (all on a B6 background), and Balb/c, CD45.1 (Ly5.1)-congenic and CD124$^{-/-}$ (both on a Balb/c background) were purchased from The Jackson Laboratory. Experiments were performed in accordance with the Swiss Federal Veterinarian Office and Cantonal Veterinary Office guidelines.

2 Cell culture

2.1 Culturing cells

NFS-60 cells and CTLL-2 cells were purchased from ATCC and cultivated in complete medium. Complete medium consists of RPMI (Invitrogen) plus 10% fetal bovine serum (Invitrogen) plus 1 mM sodium pyruvate (Invitrogen) plus 2 mM glutamate and antibiotics (Invitrogen). For NFS-60 cultivation 30 IU mIL-3 (Peprotech) and for CTLL-2 cells 100 IU hIL-2 (Proleukin, Novartis) was added to the culture.

2.2 Proliferation assay

2.2.1 Detecting murine G-CSF produced upon infection

NFS-60 cells were collected and washed 3 times in complete medium and 10^4 cells per well were seeded in a 96 well plate (flat bottom). Murine G-CSF was used as a standard. Serum with or without 500 ng/ml anti-mouse G-CSF mAb (MAB 414; R&D) was added to the wells. Cells were incubated for 24 hours and the last 4 hours 0.5 µCi ^{3}H-thymidine was added to each well. Cells were harvested (Inotech cell harvester) and transferred to a filter (Perkin Elmer). Readout occurred with a beta-counter (Wallac Trilux 1450).

2.2.2 Half-live of IL-4 after in vivo administration

CTLL-2 cells were collected and washed 3 times in complete medium and 10^4 cells per well were seeded in a 96 well plate (flat bottom). Murine IL-4 was used as a standard. Serum of previously with IL-4 intravenously treated mice was added to the wells. Cells were incubated for 72 hours and the last 4 hours 0.5 µCi ^{3}H-thymidine was added to each well. Cells were harvested (Inotech cell harvester) and transferred to a filter (Perkin Elmer). Readout occurred with a beta-counter (Wallac Trilux 1450).

2.3 Migration assay

Bone marrow cells were isolated from mice euthanized through CO2 inhalation and a positive selection for neutrophils ($Ly6G^+$ $CD11b^+$) was carried out by using Ly6G microbeads (Miltenyi Biotec). Cells were preincubated with the indicated cytokines for 20 min in RPMI plus 5% FBS. 10^5 cells were seeded in a 5 µm transwell (Corning Costar), which then was placed in media containing the cognate chemokine (CXCL1 or CXCL2; Peprotech) in the indicated concentrations. Where indicated, cells were pre-incubated for 20 min with different cytokines (30 µg/ml if not stated differently) including mIL-2 (eBioscience), mIL-4, hIL-7 or mIL-13 (all Peprotech). For pathway inhibition, SB203580 (30 µmol, Calbiochem) or Ly294002 (30 µmol, Calbiochem) were included during pretreatment. Migration occurred generally for 2 hours (except if labeled differently), then inserts were removed and cells localized in the lower compartment were counted by using a counting chamber.

3 In vivo assays

For high dose IL-4 treatment, carrier-free murine IL-4 (eBioscience) was injected intraperitoneally (i.p.) at a dose of 7.5 µg / mouse once a day for three consecutive days. IL-4 complexes were formed by mixing 1.5 µg mIL-4 with 7.5 µg anti-mIL-4 (11B11, bioXcell) and as well injected over 3 days once per day.

For high dose human G-CSF (hG-CSF) treatment, 6 µg hG-CSF (Neupogen®, Amgen) was injected once a day i.p. for three consecutive days. G-CSF complexes were formed by mixing 1 µg hG-CSF

with 6 µg anti-hG-CSF (BVD11-37610, Southern Biotech) and as well injected over 3 days once per day.

To neutralize endogenous cytokine productions, anti-IL-4 antibody (11B11) or anti-mG-CSF antibody (MAB414) were administered at a dose of 100 µg/mouse by i.p. injection once per day. To block activity of p38 MAPK in vivo, mice were given once per day for three days i.p. injections of 300 µg SB203580 (Calbiochem).

4 Airpouch model

Airpouches were generated in the back of mice by subcutaneous injection of 4 ml sterile air at day 0 and day 3. Injection of either 1 mg Monosodium Urate (MSU) crystals or 10 ng IL-1β (Peprotech) in 1 ml sterile PBS into the airpouch occurred at day 6, 15 min after intravenously injection of PBS or IL-4cx [84, 93]. After overnight migration, mice were euthanized and airpouches were flushed with PBS plus 2 mM EDTA to collect infiltrated cells. The airpouch content was counted and stained for neutrophils.

5 Flow cytometry

5.1 Cell preparation

5.1.1 Spleen

Spleens were isolated from CO_2 euthanized mice and immediately stored in cold FACS buffer (PBS plus 2% FBS plus 1mM EDTA (Milipore)). Organs were smashed through 40 µm filters in order to obtain single cell suspension. Red blood cells were lysed (Red blood cell lysing buffer, Sigma) for 2 min at room temperature, followed by extensive washing with FACS buffer. Cell counts were determined by automated cell counter (TC20 Automated cell counter, BioRad).

5.1.2 Blood

Blood was isolated immediately after death by CO_2 through cardiac puncture and stored in precooled heparinated PBS (Bichsel; 10 IE/ml). After centrifugation and discarding supernatant, pellets were lysed 2-3 times for 5 min with lysis buffer, followed by extensive washing.

5.1.3 Bone marrow

Femur und fibula were collected from CO_2 euthanized mice and stored in PBS. Bone marrow was flushed with a 29G syringe and FACS buffer and afterwards centrifuged and lysed for 2 min with lysis buffer. After extensive washing cell numbers were determined with an automated cell counter.

5.1.4 Skin

Fat tissue and hair were removed from skin which was then cut into small pieces and incubated for 1 hour at 37°C with an enzymatic cocktail consisting of 5 µg/ml Liberase TM (Roche), 1 µg/ml DNAase I (Sigma) and 5 µg/ml Dispase II (Roche) added to RPMI media. After incubation cells were liberated through extensive pipetting, filtered through a 40 µm filter and washed in FACS buffer. Cell numbers were determined by automated cell counter.

5.2 Staining antibodies

Isolated cells were surface stained using the following fluorochrome-labeled mAbs: anti-mouse CD3 (145-2C11, BD Bioscience), anti-mouse CD4 (GK1.5, eBioscience), anti-mouse CD11b (M1/70, eBioscience), anti-mouse CD45.1 (A20, eBioscience), anti-mouse CD45.2 (104, eBiosience), anti-mouse Ly6C (AL-21, BD), anti-mouse Ly6G (1A8, eBioscience), anti-mouse Gr-1 (RB6-8C5, eBiosience), anti-mouse CD11c (N418S, BioLegend), anti-mouse CD19 (1B13, eBioscience), anti-mouse MHC-II (M5/114.15.2, eBiocience), anti-mouse NK1.1 (PK136, eBioscience), anti-Annexin V (BD).

For receptor staining the following mAbs were used: anti-mouse CD124 (mIL4R-M1, BD), anti-mouse CD132 (TUGm2, BioLegend), anti-mouse IL-13Rα1 (13MOKA, eBioscience), anti-mouse CXCR2 (TAB2164P, R&D) and anti-mouse CXCR4 (2B11, BD).

For phospho-staining the following mAbs were used: anti-mouse pSTAT6 (pY641, BD), anti-mouse pp38 (pT180, pY182, BD).
Antibodies were directly conjugated to the following fluorochromes: FITC, PE, Percp-Cy5.5, APC, V450, V510, APC-Cy7, BUV395, BV711.
Cells were acquired on a BD FACSCanto II or BD LSR Fortessa flow cytometer and analyzed using FlowJo software (Tristar Inc.).

5.3 Staining

5.3.1 Surface staining

For conventional surface staining $1.5*10^6$ cells were seeded in a V-bottom plate (Costar), followed by incubation in the dark at 4°C for 15 min with the staining antibodies. Cells were washed afterwards 2 times before fixing with FACS buffer containing 1.5% paraformaldehyde (PFA; Sigma).

5.3.2 Receptor staining

For receptor staining (CD124, CD132, IL-13Rα1, CXCR2 and CXCR4) $1.5*10^6$ cells were seeded in a V bottom plate followed by incubation in the dark at room temperature for 30 min with the staining antibodies. Cells were washed afterwards 2 times before resuspending in FACS buffer and immediate analysis on a flow cytometer.

5.3.3 P38 and STAT phospho-staining

For in vitro phospho-stainings splenocytes were prepared as described above and $1.5*10^6$ cells per stain were incubated for 1 hour in RPMI medium plus 5% FBS to achieve complete dephosphorylation. After stimulation with cytokines cells were immediately fixed by addition of 10% PFA to obtain a final concentration of 1.5%. After 10 min fixation at room temperature 1 ml of ice cold methanol was added to the cells, followed by vigorous vortexing and incubation for 30 min in the fridge. Cells were then rehydrated by washing two times with FACS buffer.

Staining of surface molecules and phosphorylated antigens occurred for 30 min at room temperature in the dark.

To assess in vivo phosphorylation, mice were euthanized 15 min after i.v. injection of IL-4 or IL-4 plus SB203580 and spleens were immediately smashed into FACS buffer containing 1.5% PFA. Afterwards the same protocol was performed as with in vitro staining.

5.3.4 ROS

ROS production in $CD3^-$ $CD11b^+$ $Ly6G^+$ neutrophils was assessed using 1,2,3-dihydrorhodamine (DHR, Life Technologies) following stimulation for 20 min with phorbol 12-myristate 13-acetate (20 ng/ml, Sigma-Aldrich).

6 Bacteria

6.1 Listeria monocytogenes

6.1.1 Culture media

Listeria monocytogenes were grown under shaking conditions at 37°C in brain heart infusion medium (BHI; Oxoid) overnight. Bacteria were collected by centrifugation and resuspended in PBS. Aliquots were stored at -80°C. The next day 1 aliquot was thawed and plated in serial dilution on BHI agar plates (Oxoid) to determine cfu/ml.

6.1.2 Infection

An aliquot of prepared Listeria monocytogenes was thawed and diluted to a concentration of 10^6 cfu/ml. Mice were prewarmed under red light for 5 min and then injected i.v. with 100 µl bacterial suspension (corresponding to 10^5 cfu).

6.1.3 Bacterial load in organs

Mice were euthanized by CO_2 inhalation and liver was flushed by injection of ice cold PBS into the portal vein. Spleen and liver were collected and stored in ice chilled PBS followed by

smashing through a 40 µm or 70 µm sterile filter respectively with a total volume of 5 ml PBS. Subsequently 5 ml of 1% Trition-X100 (Sigma) diluted in water was added to the cell suspensions. After vigorous vortexing and 20 min incubation at room temperature serial dilutions were plated on BHI agar plates. Cfu per plate were counted after 24 hours incubation at 37°C.

6.2 Streptococcus

6.2.1 Culture media

Group A Streptococcus M1T1 5448 strain (GAS M1) was propagated in Todd Hewitt Broth (THB; BD) supplemented with 0.1% yeast extract (THY; Oxoid) and grown at 37°C in a static incubator.

6.2.2 Infection

For experimental procedures, overnight cultures were diluted 1:10 in THY in the morning and grown to an optical density at 600nm (OD_{600}) of 0.4. Bacteria were pelleted by centrifugation, washed twice with PBS and resuspendend in PBS. OD_{600} was adjusted to 1.2-1.3 corresponding approximately to $6*10^8$ cfu/ml. This culture was mixed 1:1 with sterile cytodex beads (Sigma) and an inoculum of $3*10^7$ cfu of GAS M1 was injected subcutaneously into the shaved flank of the mice [94, 95].

6.2.3 Bacterial load in organs

Mice were euthanized by CO_2 inhalation and skin was harvested and homogenized with a tissue lyser (Qiagen). Organ homogenates were centrifuged; supernatant was serially diluted in PBS and plated on THY plates for enumeration of viable bacteria.

7 Bone marrow chimeras

Bone marrow cells of wild-type CD45.1-congenic and $CD124^{-/-}$ CD45.2-congenic mice were purified by negative selection using magnetic beads (StemCell Technologies) and biotinylated mAbs against CD19, CD3, MHC II, NK1.1 and Ter119 (TER119, eBioscience). Lin^- bone marrow

cells from wild-type and CD124$^{-/-}$ mice were mixed and injected i.v. into irradiated (950 rad) wild-type CD45.1-congenic host mice. BM chimeric mice were kept for two weeks with 1 mg/ml Sulfamethoxazol and 0.2 mg/ml Trimethoprim (Bactrim®; Roche) in their drinking water and left untreated in order to allow for reconstitution of neutrophils before use.

8 Statistics

Differences between groups were examined for statistical significance by using student t-test or one-way analysis of variance (ANOVA) with Bonferroni's post-test correction (Prism). Data representative of one out of several experiments are displayed as mean ± standard deviation (SD), pooled data as mean ± standard error of the mean (SEM).

V RESULTS

1 Effect of IL-4cx

1.1 Effect of IL-4cx on granulocytes

G-CSF is known to expand and mobilize myelomonocytic cells, characterized by the markers CD11b and Gr-1, from the bone marrow to the circulation and subsequently the spleen [22, 28]. We analyzed these compartments in C57BL/6 (B6) wild-type mice following administration of human G-CSF (hG-CSF) in the form of recombinant hG-CSF / anti-hG-CSF monoclonal antibody (mAb) complexes (G-CSFcx) in order to deliver a prolonged G-CSF signal in vivo [96]. Notably, hG-CSF is able to stimulate mouse leukocytes [97]. Treatment with G-CSFcx led to a considerable expansion of $CD11b^+$ $Gr\text{-}1^+$ cells in the bone marrow, followed by an even more prominent increase of these cells in blood and spleen (**Figure 10**). Surprisingly, co-administration of murine IL-4 (mIL-4), in the form of mIL-4 / anti-mIL-4 mAb complexes (IL-4cx) together with G-CSFcx antagonized G-CSFcx-mediated increase of $CD11b^+$ $Gr\text{-}1^+$ cells in the bone marrow, blood and spleen (**Figure 10**).

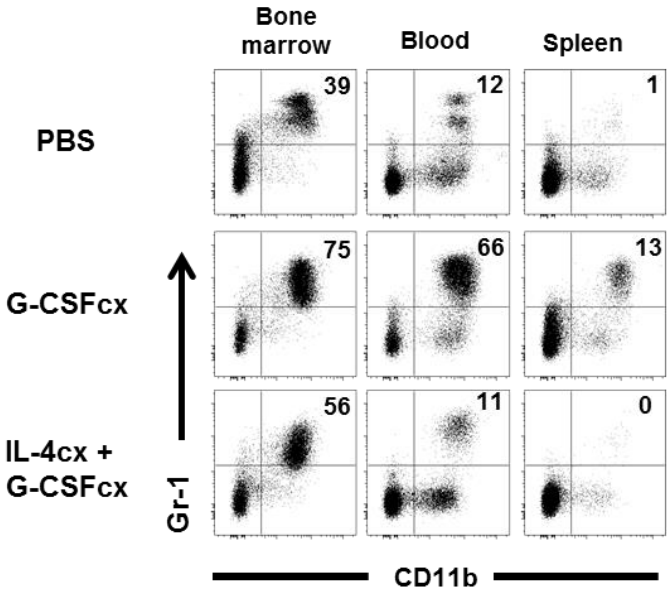

Figure 10. IL-4cx antagonizes G-CSFcx-mediated expansion of granulocytes in bone marrow and periphery. B6 wild-type mice were treated with PBS, G-CSFcx, or G-CSFcx plus IL-4cx for 3 consecutive days. Bone marrow, blood and spleen were analyzed 16 hours after the last injection. Shown are $CD3^-$ cells for their expression of Gr-1 and CD11b. Plots are representative of 2 experiments with a total of 4 mice.

1.2 Effect of IL-4cx on neutrophils

Because $CD11b^+$ $Gr\text{-}1^+$ cells contain both monocytes and neutrophils, we decided to distinguish these cell subsets by using the markers Ly6C and Ly6G, which are specific for monocytes and neutrophils, respectively [22]. Administration of G-CSFcx to mice caused mainly expansion of $CD11b^+$ $Ly6G^+$ neutrophils in the bone marrow, leading to a marked increase of neutrophils in blood and spleen of these mice, both in percentages (**Figure 11A**) and cell counts (**Figure 11B**). Injection of IL-4cx together with G-CSFcx abolished the effect of G-CSFcx on neutrophils in the bone marrow, and, as seen with $CD11b^+$ $Gr\text{-}1^+$ cells, IL-4cx blocked as well G-CSFcx-mediated accumulation of neutrophils in blood and spleen leading to neutrophil percentages and counts comparable to PBS-treated control mice (**Figure 11A** and **11B**). These data suggest an important effect of IL-4cx in controlling the neutrophil counts in periphery.

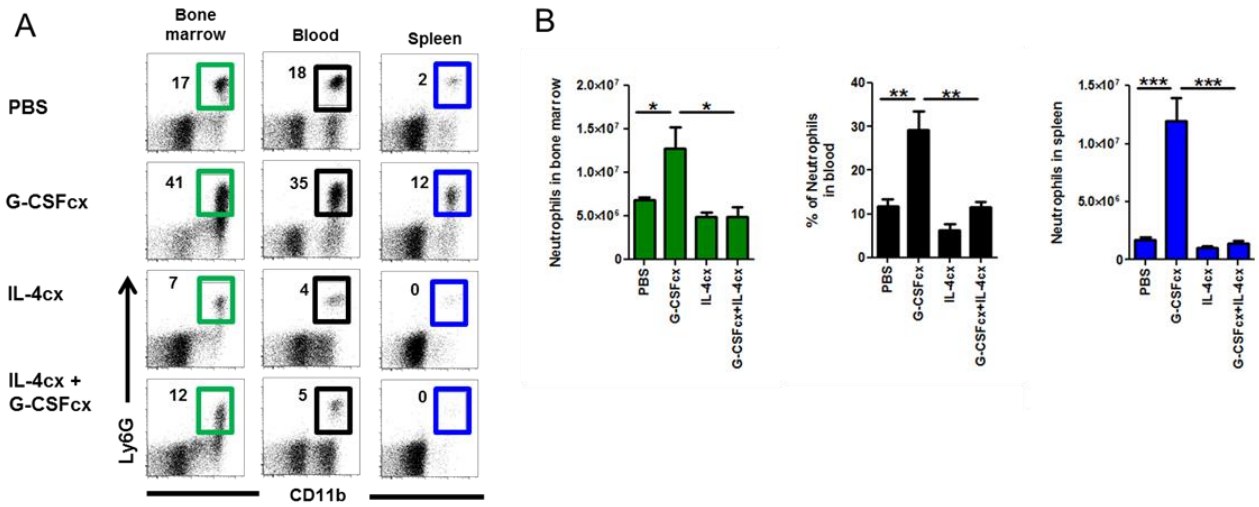

Figure 11. IL-4cx antagonizes G-CSFcx-mediated expansion of neutrophils in bone marrow and periphery. B6 wild-type mice were treated with PBS, G-CSFcx, IL-4cx, or G-CSFcx plus IL-4cx for 3 consecutive days. Bone marrow, blood and spleen were analyzed 16 hours after the last injection. (**A**) Shown are $CD3^-$ cells for their expression of Ly6G and CD11b and (**B**) quantification of $CD11b^+$ $Ly6G^+$ neutrophils in the indicated organs. Plots are representative of one out of three experiments with 2-3 mice per group; quantifications are pooled from 3 independent experiments with a total of 7 mice per group. **P*<0.05; ***P*<0.01; ****P*<0.001.

1.3 Effect of IL-4cx on other immune cells

In contrast to $CD11b^+$ $Ly6G^+$ neutrophils, percentages (**Figure 12A**) and cell counts (**Figure 12B**) of $CD11b^+$ $Ly6G^-$ $Ly6C^+$ monocytes were not or only minimally affected by administration of G-CSFcx, or G-CSFcx plus IL-4cx.

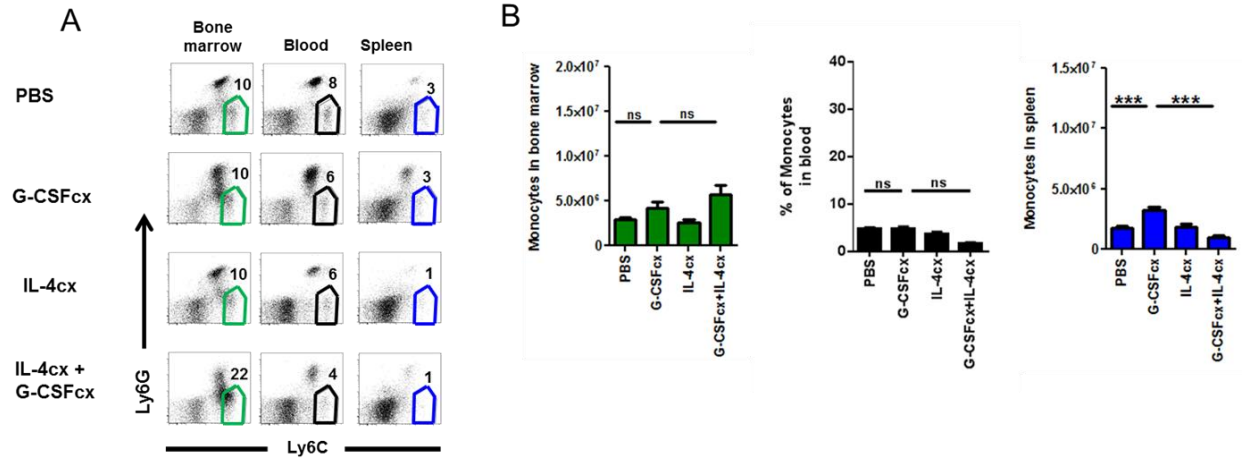

Figure 12. IL-4cx only affects minimally monocytes. B6 wild-type mice were treated with PBS, G-CSFcx, IL-4cx, or G-CSFcx plus IL-4cx for 3 consecutive days. Bone marrow, blood and spleen were analyzed 16 hours after the last injection. (**A**) Shown are $CD3^-$ $CD11b^+$ cells for their expression of Ly6G and Ly6C and (**B**) quantification of $CD11b^+$ $Ly6C^+$ monocytes in the indicated organs. Plots are representative of one out of three experiments with 2-3 mice per group; quantifications are pooled from 3 independent experiments with a total of 7 mice per group. ****P*<0.001; ns = not significant.

Unlike neutrophils, counts of other cell populations, such as B cells (**Figure 13A**) and $CD4^+$ T cells (**Figure 13B**) were not significantly reduced by G-CSFcx or G-CSFcx plus IL-4cx. However, IL-4cx by themselves showed a minimal increase in $CD4^+$ T cells. Natural killer (NK) cells reacted to G-CSFcx and IL-4cx separately by showing a marginal increase in cell number, however no synergistic or antagonistic effect was observed (**Figure 13C**). Conventional dendritic cells, characterized by the expression of CD11c and MHCII were, comparable to $CD4^+$ T cells, stimulated by IL-4cx (**Figure 13D**).

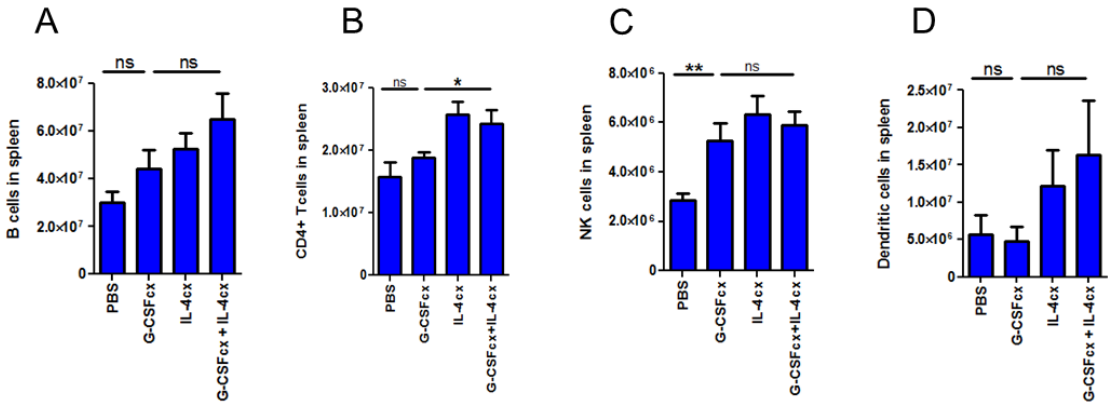

Figure 13. IL-4cx does not reduce cell numbers of other immune cells. B6 wild-type mice were treated with PBS, G-CSFcx, IL-4cx, or G-CSFcx plus IL-4cx for 3 consecutive days. Spleen was analyzed 16 hours after the last injection. Shown are cell counts of (**A**) $CD3^-$ $CD19^+$ B cells, (**B**) $CD3^+$ $CD4^+$ T cells, (**C**) $CD3^-$ $NK1.1^+$ natural killer (NK) cells and (**D**) $CD11c^+$ $MHCII^+$ dendritic cells. Quantifications are pooled from 3 independent experiments with a total of 7 mice per group. *P<0.05; **P<0.01; ns = not significant.

1.4 High-dose cytokine administration mimics cytokine complexes

As mentioned earlier, by increasing the dose of injected cytokine its in vivo effects can be extended. Treating B6 wild-type mice with high doses of cytokines led in all the three compartments of interest to a similar increase in neutrophil percentage (**Figure 14A**) and counts (**Figure 14B**).

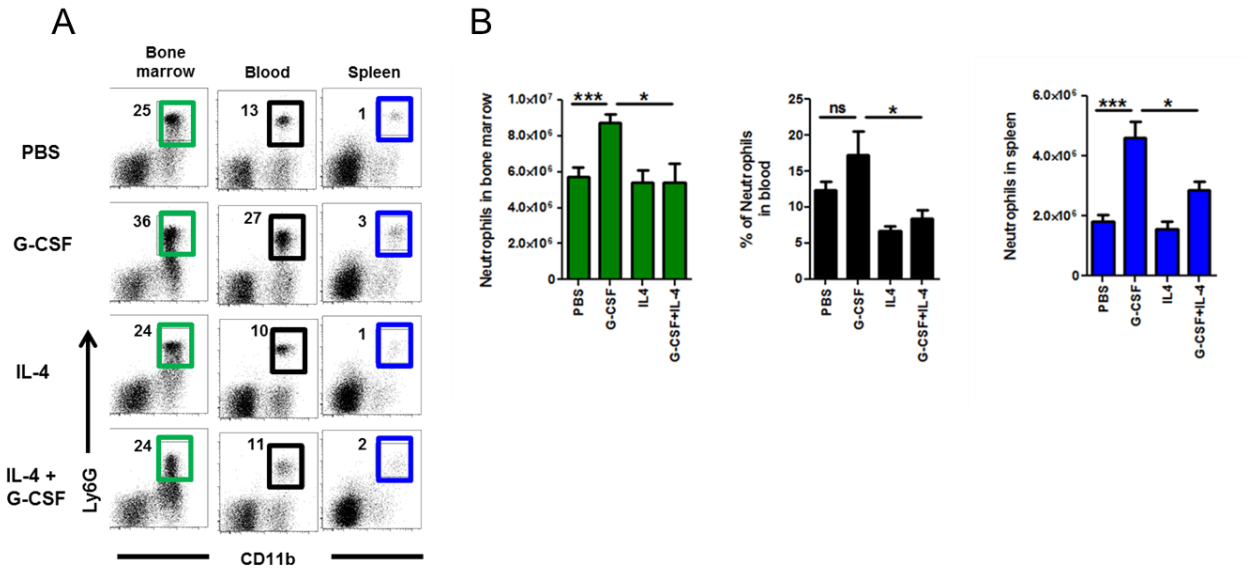

Figure 14. High-dose administration of cytokines replicates effects of complexed cytokines. (**A**) B6 wild-type mice were treated with PBS, G-CSF, IL-4, or G-CSF plus IL-4 for 3 consecutive days. Bone marrow, blood and spleen were analyzed 16 hours after the last injection. (**A**) Shown are $CD3^-$ cells for their expression of Ly6G and CD11b and (**B**) quantification of $CD11b^+$ $Ly6G^+$ neutrophils in the indicated organs. Plots are representative of one out of three experiments with 2-3 mice per group, quantifications are pooled from 3 independent experiments with a total of 7 mice per group. **P*<0.05; ****P*<0.001; ns= not significant.

2 Use of IL-4cx in Listeria monocytogenes infection

2.1 Production of G-CSF following acute infection

Having established a role of IL-4cx in antagonizing specifically G-CSF-mediated neutrophil expansion, we were interested if the same effect could be detected in a more physiological setting. It is known that infections lead to increased G-CSF secretion and plasma levels [58]. Therefore we investigated IL-4cx action in a model of systemic Listeria monocytogenes (LM) infection. 24 hours after intravenous infection with 10^5 colony-forming units (cfu) LM, serum was harvested and assessed for biologically active G-CSF using NFS-60 cells, a cell line responding to murine G-CSF [98]. Uninfected wild-type mice served as controls. Serum from infected mice enhanced proliferation of NFS-60 cells, whereas serum from infected mice supplemented in vitro with the anti-mG-CSF neutralizing mAb MAB414 showed proliferative capacity of uninfected mice, thereby demonstrating that G-CSF was indeed produced upon infection (**Figure 15A** left panel). G-CSF produced upon infection could also be neutralized directly in vivo by injection of anti-mG-CSF mAb (MAB414) 1 day prior to infection and on the day of infection. Serum of these G-CSF-neutralized mice showed similar proliferation capacity as serum of uninfected mice (**Figure 15A** left panel).

Similar to injection of G-CSFcx, infection with 10^5 cfu LM led to recruitment of $CD11b^+$ $Ly6G^+$ neutrophils to the periphery, which was dependent on endogenous G-CSF production, as evidenced by an absence of neutrophil mobilization upon treatment with neutralizing anti-mG-CSF mAb (**Figure 15B and 15C**).

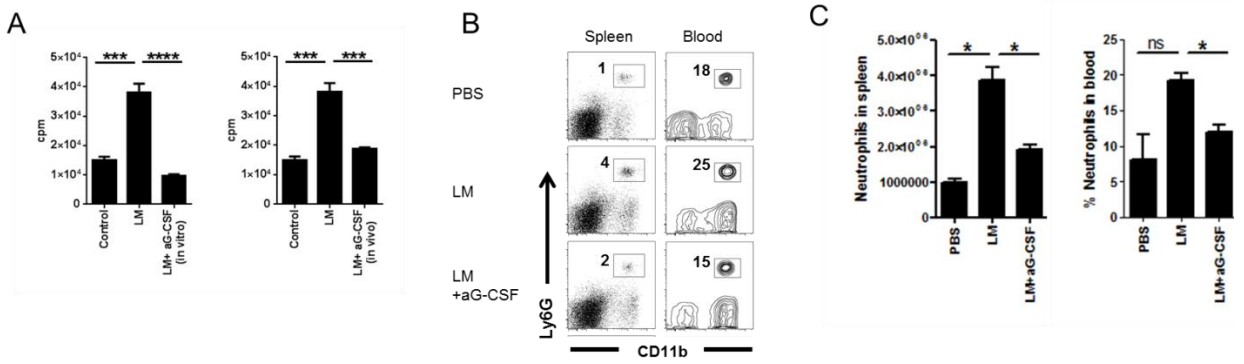

Figure 15. G-CSF is produced upon acute Listeria monocytogenes infection. (**A**) Left panel: proliferation of NFS-60 cells (G-CSF sensitive cell line) in response to serum from uninfected B6 mice or from mice infected with 10^5 colony forming units (cfu) LM 24 hours prior to serum sampling, followed by in vitro addition of anti-G-CSF where indicated. Right panel: B6 mice received either PBS or 10^5 cfu LM with or without anti-G-CSF mAb (aG-CSF) on days -1 and 0 of infection, followed by serum analysis 24 hours later. Shown are (**B**) flow cytometry analysis of $CD3^-$ $CD11b^+$ $Ly6G^+$ neutrophil frequencies in spleen and blood as well as (**C**) neutrophil counts in spleen and blood. Data are representative of 2 independent experiments with a total of 4-5 animals per condition. *P<0.05; ***P<0.001; ****P<0.0001; ns = not significant.

2.2 IL-4cx antagonizes endogenous G-CSF

We also used the model systemic LM infection to investigate the in vivo consequences of IL-4cx signals on G-CSFcx-mediated expansion and recruitment of neutrophils. Mice treated for 3 days prior to infection with IL-4cx did not show an increase in neutrophils in blood or spleen 24 hours after infection (**Figure 16A**). In contrast to PBS-treated control mice succumbing to 10^5 cfu LM by 4 days following infection, animals pretreated for 3 days with G-CSFcx all survived this challenge. Strikingly, co-injection of IL-4cx and G-CSFcx led to a loss of the beneficial effect, both in terms of weight loss and survival (**Figure 16B and 16C**). The beneficial effect of G-CSFcx is abolished when neutrophils are depleted by using Ly6G specific depleting antibody (clone 1A8) (**Figure 16D**). Pretreatment of wild-type mice with G-CSFcx led already after 24 hours - and even more prominent after 72 hours - to a considerable decrease of bacterial load in spleen. A similar tendency was observed in liver, although this difference did not reach statistical significance probably due to group size. In line with the survival data, bacterial load was comparable to PBS-treated mice when IL-4cx was co-administered with G-CSFcx (**Figure 16E**).

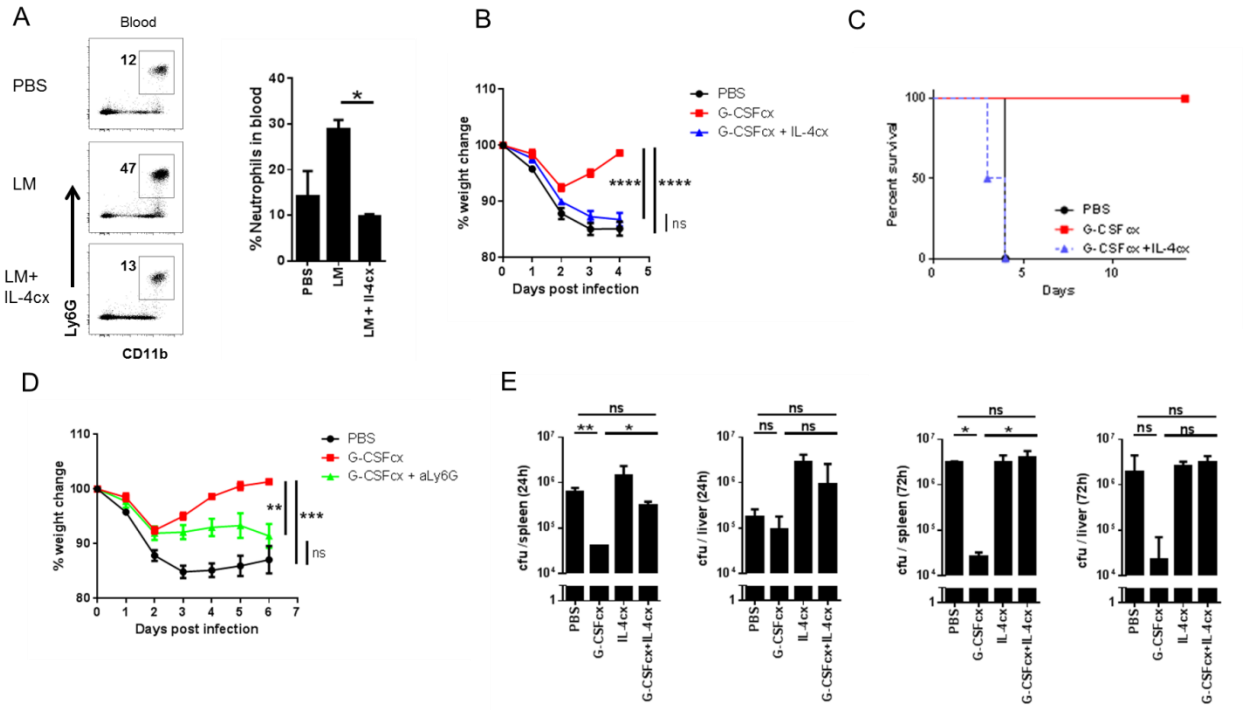

Figure 16. IL-4cx antagonizes beneficial effects of infection-induced G-CSF secretion. (**A**) B6 mice received either PBS or 10^5 cfu LM intravenously (i.v.) without or with pretreatment using IL-4cx on days -3 to -1 prior to infection, followed by analysis 24 hours later. Shown is flow cytometric analysis of $CD3^-$ $CD11b^+$ $Ly6G^+$ neutrophil frequencies in blood. (**B-E**) B6 wild-type mice were pretreated with PBS, G-CSFcx, G-CSFcx plus IL-4cx or G-CSFcx plus anti Ly6G followed by systemic infection with 10^5 cfu LM, and were either (**B and D**) monitored for weight change, (**C**) survival or (**E**) assessed for LM cfu in spleen and liver 24 hours and 72 hours post infection. Data are representative of 2-3 independent experiments with a total of 4-5 animals per condition or (B and D) pooled from 2 experiments with n=6. **P*<0.05; ****P*<0.001; *****P*<0.0001; ns = not significant.

3 IL-4cx act directly on neutrophils

3.1 IL-4cx in RAG$^{-/-}$ animals

As stated before, IL-4 can be stimulatory for many different cell types, including B and T cells. Therefore the effect on neutrophils could as well be due to secondary factors released from these cells upon IL-4 stimulation. Mice deficient in recombination-activating gene (RAG) lack mature T and B cells [99]. Interestingly, IL-4cx was able to counterbalance the effects of G-CSFcx

on neutrophils in both wild-type and RAG$^{-/-}$ mice, indicating that neither mature T nor B cells nor T or B cell-derived cytokines were crucial for the observed effects (**Figure 17A and 17B**).

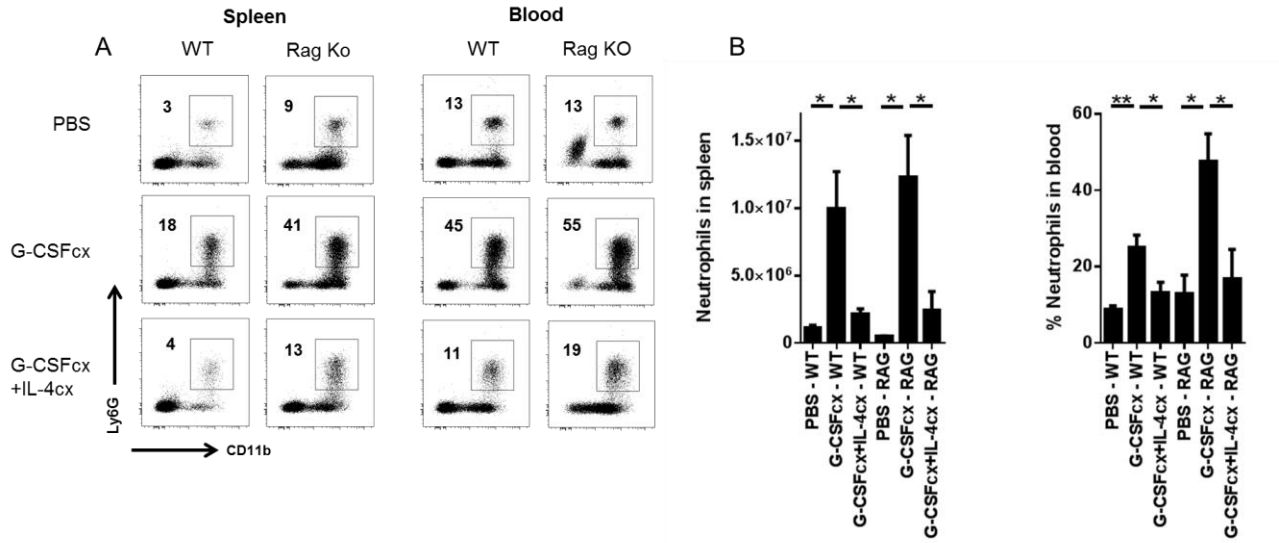

Figure 17. Mature B and T cells are dispensable for IL-4cx effects on neutrophils. B6 wild-type or RAG1-deficient mice (Rag KO) were treated with PBS, G-CSFcx, or G-CSFcx plus IL-4cx for 3 consecutive days. Blood and spleen were analyzed 16 hours after the last injection. (A) Shown are CD3^{-} cells for their expression of Ly6G and CD11b and (B) quantification of CD11b^{+} Ly6G^{+} neutrophils in the indicated organs. Plots are representative and quantification pooled from two experiments with a total of 3-4 mice per group. *P<0.05; **P<0.01.

3.2 Generation of bone marrow chimeras using CD124$^{-/-}$ mice

To further investigate whether IL-4 acted directly or indirectly on neutrophils in vivo, we generated bone marrow chimeras carrying a mixture of CD124 knockout (KO) and wild-type immune cells by reconstituting lethally-irradiated WT (CD45.1) mice with a mixture of immune lineage-depleted CD45.2-congenic CD124$^{-/-}$ and CD45.1-congenic wild-type bone marrow cells (**Figure 18A**). After reconstitution, the ratio of CD45.1 (wild-type) to CD45.2 (CD124$^{-/-}$) cells was determined, followed by treatment for 3 days with either PBS, G-CSFcx, or G-CSFcx plus IL-4cx. 16 hours after the last injection, the change of ratio of CD45.2^{+} to CD45.1^{+} neutrophils was determined.

Upon administration of G-CSFcx, CD124$^{-/-}$ neutrophils showed a slightly higher expansion than their wild-type counterparts, presumably due to endogenously produced IL-4 impeding the egress of wild-type neutrophils from the bone marrow (**Figure 18B**). This effect was even more pronounced in bone marrow chimeras receiving G-CSFcx plus IL-4cx, in which wild-type neutrophils were outnumbered by their CD124$^{-/-}$ counterparts (**Figure 18B**).

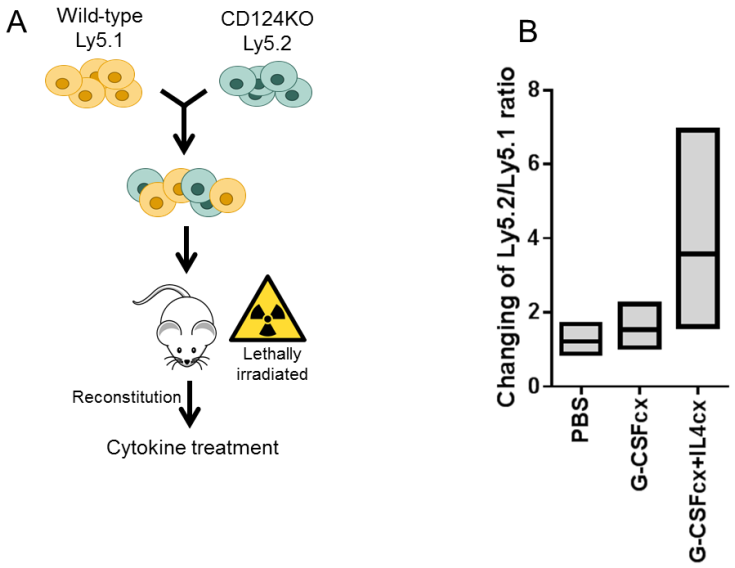

Figure 18. IL-4cx acts directly on neutrophils. (**A**) Immune cell-lineage-depleted bone marrow cells of wild-type (CD45.1$^+$; orange) and CD124-deficient (CD124KO; CD45.2$^+$; blue) mice were mixed and adoptively transferred to lethally-irradiated CD45.1$^+$ wild-type hosts. (**B**) After reconstitution, bone marrow chimeric mice were injected with PBS, G-CSF, or G-CSF plus IL-4, and the change in ratios of CD45.2$^+$ to CD45.1$^+$ cells within CD3$^-$ CD11b$^+$ Ly6G$^+$ blood neutrophils was determined by flow cytometry 16 hours after the last injection.

4 The role of IL-4 receptors in IL-4-mediated neutrophil inhibition

4.1 CD124 is crucial for IL-4cx-mediated effects

As mentioned in the introductory part of this thesis, IL-4 can signal through two different receptors, and both contain CD124 (IL-4Rα). In a first experiment, we confirmed that the effect of IL-4 is indeed mediated through CD124. Wild-type and CD124-deficient mice, both on a Balb/c background, were treated for three consecutive days with PBS, G-CSFcx, or G-CSFcx plus IL-4cx followed by analysis of the neutrophil compartment in blood and spleen (**Figure 19A** and **19B**). G-CSFcx were able to potently expand neutrophils in both mouse strains, however, IL-4cx antagonized the effect of G-CSFcx only in wild-type mice, but not in CD124-deficient animals. Wild-type Balb/c mice systemically infected with 10^5 cfu LM succumbed to the infection by day 4 after infection, whereas all CD124KO mice survived the bacterial challenge (**Figure 19C**). This indicated that signaling via the IL-4R negatively impacted survival of mice.

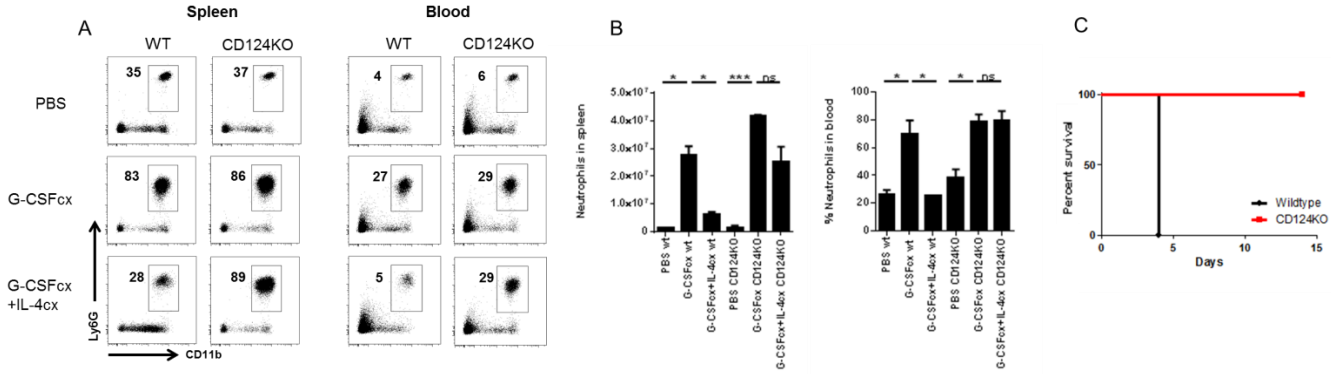

Figure 19. CD124 is crucial for IL-4cx-mediated effects on neutrophils. Wild-type and CD124-deficient (CD124KO) mice, both on a Balb/c background, were treated with PBS, G-CSFcx, or G-CSFcx plus IL-4cx for 3 consecutive days. Spleen and blood were analyzed 16 hours after the last injection. (**A**) Shown is expression of CD11b and Ly6G in $CD3^-$ cells and (**B**) quantification of $CD11b^+$ $Ly6G^+$ neutrophils in the indicated organs. Wild-type and CD124KO mice were infected with 10^5 cfu LM and analyzed for their survival. Plots are representative of one out of two experiments with each 2 mice per group; quantifications and survival are pooled from 2 independent experiments with a total of 4-6 mice per group. *P<0.05; ***P<0.001; ns= not significant.

4.2 Type I IL-4 receptor is dispensable for IL-4cx action

As signaling through CD124 could be mediated through both IL-4R types, we wanted to determine which of the IL-4Rs is responsible for the effects of IL-4cx. The type I IL-4R consists of a heterodimer of CD124 and the common gamma chain (γ_c; CD132). Mice deficient for CD132 and wild-type B6 mice were treated for three consecutive days with PBS, G-CSFcx, or G-CSFcx plus IL-4cx followed by analysis of the neutrophil compartment in blood and spleen (**Figure 20A** and **20B**). Surprisingly, we found a similar expansion of neutrophils in both mice, indicating that CD132 was not necessary for the IL-4cx-mediated effect on neutrophils.

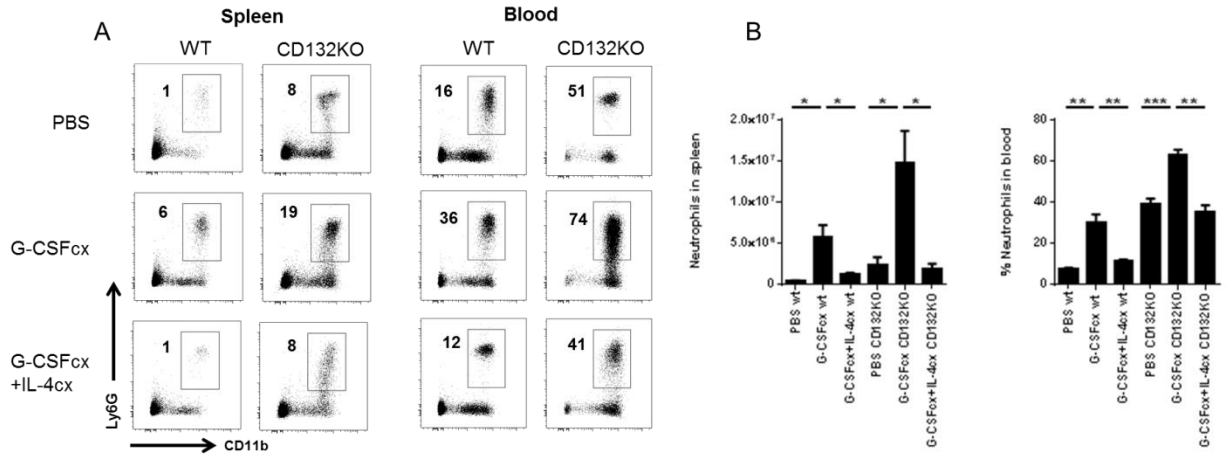

Figure 20. CD132 is dispensable for IL-4cx-mediated effects on neutrophils. B6 wild-type and CD132 deficient (CD132KO) mice were treated with PBS, G-CSFcx, or G-CSFcx plus IL-4cx for 3 consecutive days. Spleen and blood were analyzed 16 hours after the last injection. (**A**) Shown are $CD3^-$ cells for their expression of CD11b and Ly6G and (**B**) quantification of $CD11b^+$ $Ly6G^+$ neutrophils in the indicated organs. Plots are representative of one out of two experiments with each 2 mice per group; quantifications are pooled from 2 independent experiments with a total of 4 mice per group. **P*<0.05; ***P*<0.01; ****P*<0.001.

4.3 IL-4 receptor regulation

Originally it was believed that the type II IL-4R is mainly present on non-hematopoietic cells [73]. However, our data suggest that the IL-4-mediated effects on neutrophils are rather dependent on type II IL-4Rs. Bone marrow neutrophils showed high expression levels of IL-13Rα1, whereas their expression of CD124 and CD132 was rather low (**Figure 21A**). Upon

treatment with G-CSFcx, CD124 was upregulated and the expression levels of IL-13Rα1 even further increased, while CD132 remained at background levels (**Figure 21A-21C**). Neutrophils circulating in the blood showed higher expression of IL-13Rα1 than in the bone marrow and, upon G-CSFcx treatment, this expression further increased. On the other hand, CD132 was unaffected by G-CSFcx treatment, both in bone marrow and spleen, whereas only circulating blood neutrophils showed a minimal increase in CD132 expression (**Figure 21A** and **21D**).

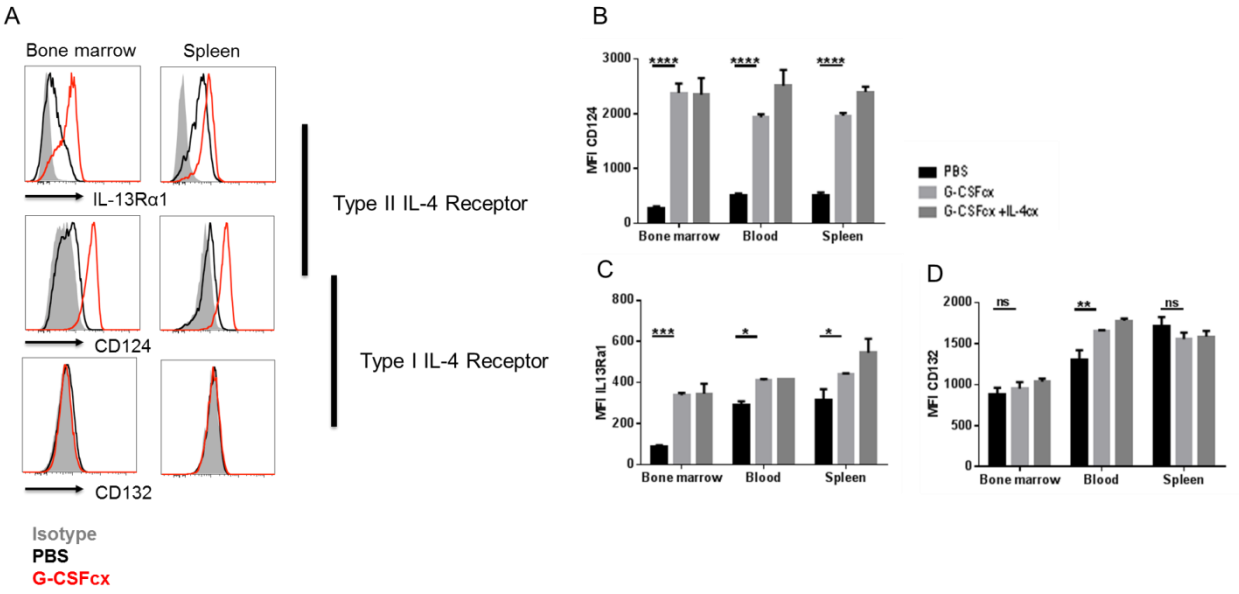

Figure 21. Type II IL-4 receptor subunits are upregulated upon G-CSFcx stimulation. (**A**) Histograms showing IL-4R subunit expression compared to isotype control (grey shaded area) after 3 injections of PBS (black line) or G-CSFcx (red line) in B6 wild-type mice. Quantification of (**B**) CD124, (**C**) IL-13Rα1 and (**D**) CD132 expression. Histograms and plots are representative of one out of 2 experiments, each with 2-3 mice per group. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001; ns = not significant.

5 Chemokine receptor expression on neutrophils

As mentioned previously, the chemokine receptor CXCR4 mediates retention of neutrophils in the bone marrow [34, 37]. The expression of CXCR4 was affected in bone marrow neutrophils differently upon G-CSFcx or IL-4cx treatment. As expected, administration of G-CSFcx led to downregulation of CXCR4, therefore leading to higher neutrophil counts in periphery. On the other hand, IL-4cx led to a significant upregulation of CXCR4, thereby increasing the cells'

responsiveness to retention signals in the bone marrow (**Figure 22A** and **22B**). After a 3-day course of cytokine injection CXCR4 expression in spleen was not significantly altered (**Figure 22B**).

CXCR2 on the other hand is an important chemotactic receptor in mediating neutrophil migration towards CXCR2-binding chemokines. In our experimental system, G-CSFcx led to a significant upregulation of CXCR2 on bone marrow neutrophils, thereby facilitating their egress into the periphery (**Figure 22C** and **22D**). IL-4cx, however, was only affecting CXCR2 expression on neutrophils in the periphery by downregulation of CXCR2 on these cells, thereby rendering them most likely less sensitive towards chemoattraction in the periphery (**Figure 22D**).

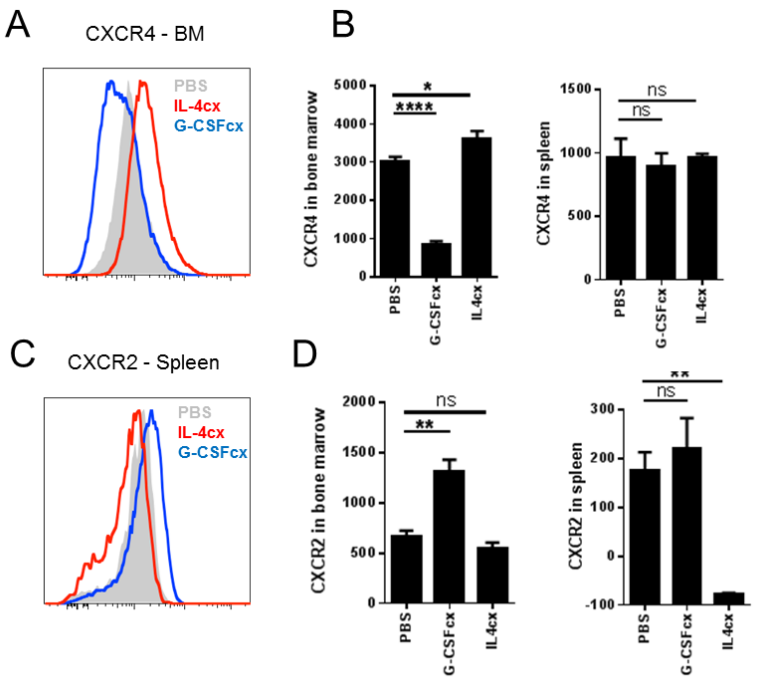

Figure 22. G-CSFcx and IL-4cx alter expression of CXCR2 and CXCR4 on neutrophils. B6 wild-type mice were treated with PBS, G-CSFcx or IL-4cx for 3 consecutive days. Bone marrow and spleen were analyzed 16 hours after the last injection. Histogram showing (**A**) CXCR4 expression in bone marrow or (**C**) CXCR2 expression in $CD3^{-}$ $CD11b^{+}$ $Ly6G^{+}$ neutrophils from spleen and quantification of mean fluorescence intensity of (**B**) CXCR4 or (**D**) CXCR2 in neutrophils from bone marrow and spleen. Plots are representative from one out of two experiments with each 2 mice per group; **P*<0.05; ***P*<0.01; *****P*<0.0001; ns = not significant.

6 Neutrophil maturation

As mentioned in the introduction, reduced neutrophil counts in the periphery could be due to altered generation and egress from the bone marrow but also due to enhanced apoptosis. We cultured bone marrow cells for 24 hours in vitro in the presence of PBS, G-CSF, IL-4, or G-CSF plus IL-4, and determined the percentage of Annexin V^+ cells within $CD11b^+$ $Ly6G^+$ neutrophils. As expected, G-CSF protected neutrophils from apoptosis, whereas IL-4 alone did not show any effect. Importantly neutrophils stimulated with G-CSF plus IL-4 showed an enhanced rate of apoptosis (**Figure 23A**). Simultaneously, Ly6G expression on neutrophils, known to be high on more mature neutrophils [100], was elevated upon stimulation with G-CSF plus IL-4 (**Figure 23B**). Presumably, the $Ly6G^{high}$ population became apoptotic, whereas in neutrophils expressing intermediate levels of Ly6G ($Ly6G^{int}$) only minimal Annexin V^+ cells were detected (**Figure 23C**).

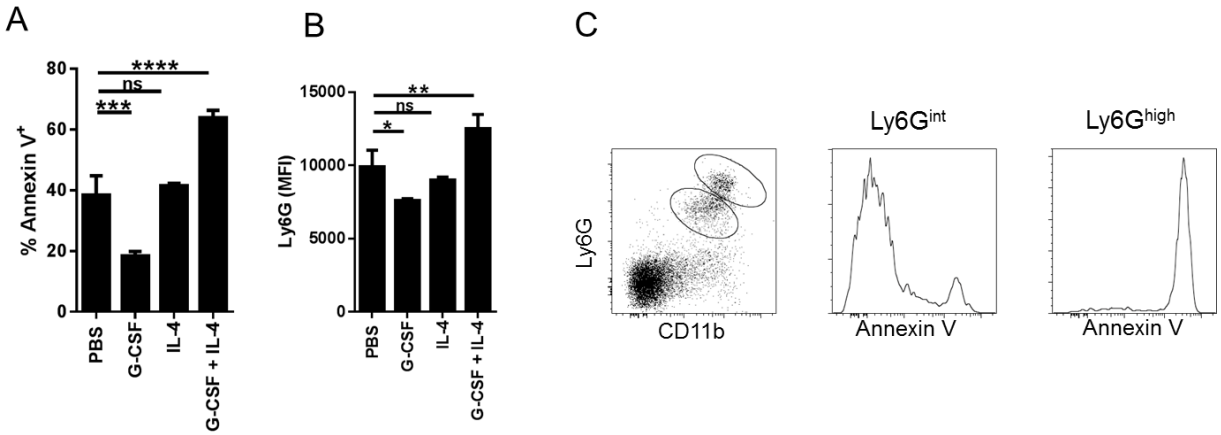

Figure 23. Combination of G-CSF and IL-4 enhances neutrophil maturation and apoptosis. Bone marrow was isolated from B6 wild-type mice and cultured for 24 hours in vitro with PBS, G-CSF, IL-4, or G-CSF plus IL-4. (**A**) Quantification of Annexin V^+ cells and (**B**) mean fluorescene intensity of Ly6G in $CD11b^+$ $Ly6G^+$ neutrophils. (**C**) Flow cytometric analysis of PBS-stimulated neutrophils and histograms showing Annexin V levels in $Ly6G^{int}$ and $Ly6G^{high}$ expressing cells. Data are representative of one out of two experiments with n = 2 or 3; **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001; ns = not significant.

7 Effect of IL-4 on neutrophil migration

7.1 Effect of IL-4 on neutrophil migration in vitro

7.1.1 Effect of IL-4 on in vitro migration of wild-type neutrophils

The effects of IL-4cx on dampening neutrophil expansion in the periphery upon G-CSF signals could also be due to a failure of neutrophils to migrate out of the bone marrow and into the tissues, as also suggested by an altered expression pattern of CXCR2 and CXCR4. Thus, we next tested whether IL-4 was interfering with neutrophil migration.

Neutrophils have the capacity to migrate towards the CXCR2-binding chemokines CXCL1 and CXCL2. We assessed in vitro the chemotaxis of purified $CD11b^+$ $Ly6G^+$ bone marrow derived neutrophils under different conditions. Significant migration towards 100 ng/ml CXCL2 occurred already within 15 minutes, reaching a clear plateau after 2 hours of migration time (**Figure 24A**). Incubation of neutrophils for 20 minutes before seeding with a concentration of 30 ng/ml IL-4 reduced their migration capacity during the entire time period assessed. To identify the optimal dose of the chemoattractants CXCL2 (**Figure 24B**) and CXCL1 (**Figure 24C**), neutrophil migration was determined in varying concentrations of these chemoattractants, showing that 100 ng/ml induced the strongest migration in cells that had not been pretreated. Already a concentration of 3 ng/ml IL-4 was able to diminish the migration capacity of neutrophils, which was maximally reduced with 30 ng/ml IL-4 (**Figure 24D** and **24E**). Other cytokines using CD132 for their signaling pathway (such as IL-2, IL-7 and IL-15) or another Th2-type cytokine (IL-13) were not able to induce the same effect as IL-4 did (**Figure 24F**). Collectively, these data suggest that migration towards CXCR2-binding chemokines is inhibited upon direct signaling of IL-4 on neutrophils.

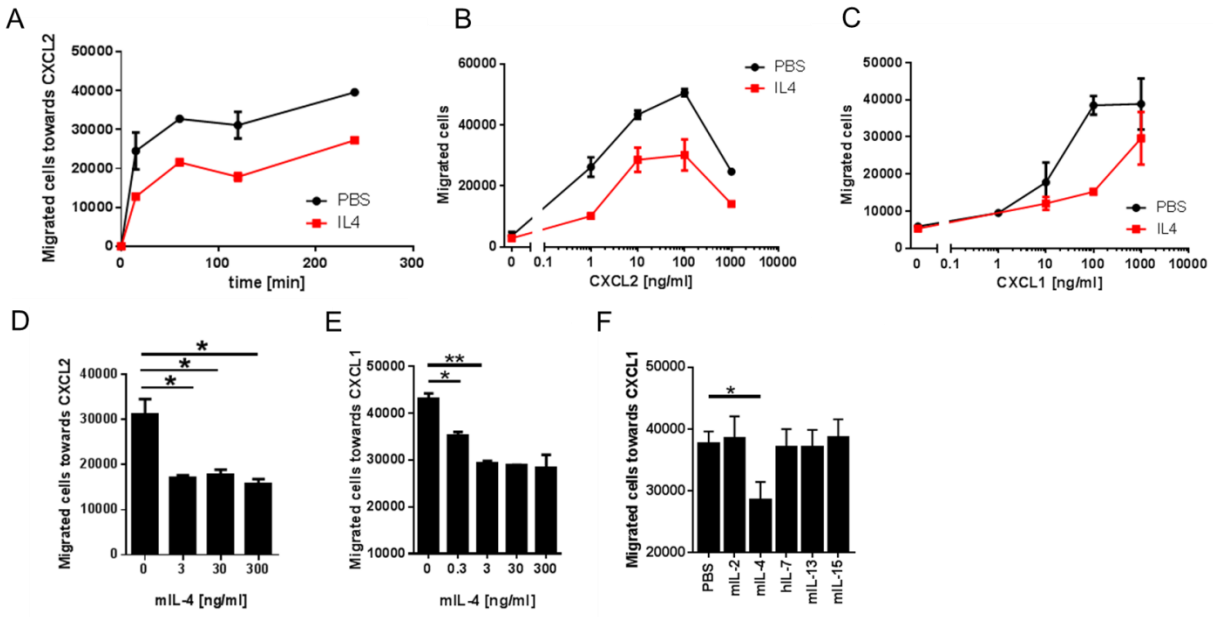

Figure 24. In vitro migration of neutrophils to CXCR2-binding chemokines is decreased by IL-4. (**A**) Migration of purified bone marrow-derived CD11b$^+$ Ly6G$^+$ neutrophils over 240 minutes in the presence of PBS or 30 ng/ml of IL-4 towards 100 ng/ml CXCL2. Migration to titrated concentration of (**B**) CXCL2 and (**C**) CXCL1 in the presence of PBS or a fixed concentration of IL-4 (30 ng/ml). Migration to a constant concentration of either (**D**) CXCL2 (100 ng/ml) or (**E**) CXCL1 (100 ng/ml) in the presence of increasing amounts of murine IL-4. (**F**) Purified neutrophils were treated in vitro with PBS, IL-2, IL-4, IL-7, IL-13 or IL-15 followed by migration towards CXCL1. Shown are total migrating neutrophils. Plots are representative (A-E) or pooled data (F) of at least two independent experiments with n = 2. *P<0.05; **P<0.01.

7.1.2 Effect of IL-4 on in vitro migration of neutrophils deficient in IL-4R subunits

Next, we determined the migration capacity of CD124-deficient neutrophils upon stimulation with IL-4. CD11b$^+$ Ly6G$^+$ neutrophils from Balb/c wild-type mice served as controls. In contrast to wild-type neutrophils, CD124$^{-/-}$ neutrophils were unable to respond to IL-4 and showed, in the presence of IL-4, no decrease of their migration rate towards CXCL2 (**Figure 25A**) and CXCL1 (**Figure 25B**).

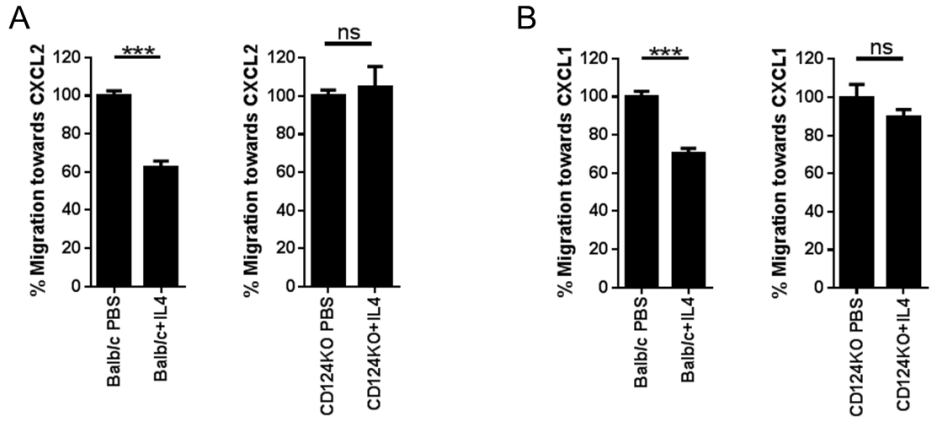

Figure 25. In vitro migration of CD124-deficient neutrophils to CXCR2-binding chemokines. Relative migration for 2 hours of purified $CD11b^{+}$ $Ly6G^{+}$ neutrophils from wild-type or CD124-deficient (CD124KO) mice to either (**A**) CXCL2 or (**B**) CXCL1 in the presence of either PBS or 30 ng/ml IL-4. Results are pooled from two independent experiments with a total of n=4; ***P<0.001; ns = not significant.

To dissect between the type I and type II IL-4Rs, we assessed the migration capacity of CD132-deficient neutrophils. Interestingly, we found that $CD132^{-/-}$ bone marrow neutrophils were unable of sensing IL-4 in the dose used as shown by a similar migration ratio as cells migrating in the absence of IL-4 (**Figure 26A and 26B**). However, by increasing the dose of IL-4 from 30 ng/ml to 500 ng/ml, also CD132-deficient cells were inhibited by IL-4 in their migration (**Figure 26B**). Moreover, neutrophils isolated from $CD132^{-/-}$ mice that had been pretreated 16 hours earlier with one injection of G-CSFcx, thereby upregulating IL-13Rα1 (**Figure 21A**), also low-dose IL-4 was able to decrease neutrophil migration (**Figure 26C**). These data indicate that the inhibition of in vitro migration by IL-4 in neutrophils is mediated at low IL-4 concentrations by type I IL-4Rs, whereas at high IL-4 doses type II IL-4Rs also contribute to this effect.

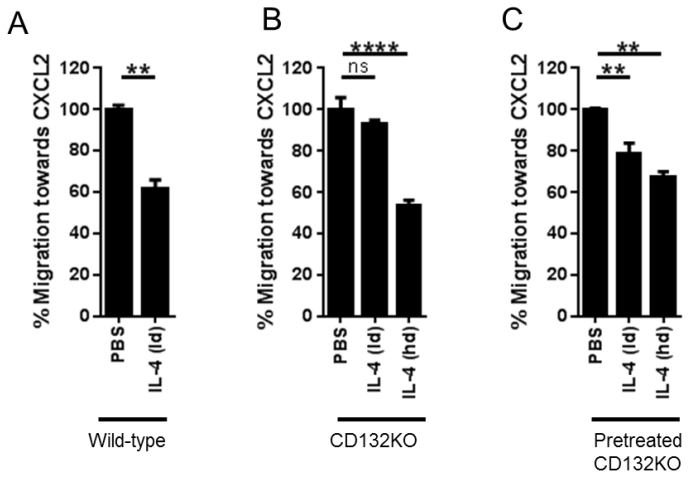

Figure 26. In vitro migration of CD132-deficient neutrophils towards CXCL2. Relative migration for 2 hours of purified $CD11b^+$ $Ly6G^+$ neutrophils from either (**A**) B6 wild-type, (**B**) CD132-deficient (CD132KO) or (**C**) CD132KO mice pretreated with G-CSFcx in vivo followed by purification of neutrophils towards CXCL2 in the presence of either PBS or 30 ng/ml (low dose; ld) IL-4 or 500 ng/ml IL-4 (high dose; hd), as indicated. Relative migration after incubation with either PBS, low-dose or high-dose IL-4 is shown. Results are pooled from two independent experiments with a total of n = 4-5; ***P*<0.01; *****P*<0.0001 ns = not significant.

7.2 Effect of IL-4 on neutrophil migration in vivo

In order to assess in vivo migration of neutrophils, we used the airpouch mouse model. 16 hours after injection of PBS (control), MSU or IL-1β into the preformed airpouch, neutrophil migration into this compartment was assessed. Upon stimulation with either MSU or IL-1β the percentage of neutrophils in the airpouch increased (**Figure 27A** and **27B**). Migration into the airpouch critically depended on CXCR2, as in CXCR2-deficient mice no neutrophils could be detected in the airpouch, although these mice contained even increased neutrophil counts in the peripheral blood already at steady state (**Figure 27A**). Pretreatment of mice using i.v. injection of IL-4cx 15 minutes before initiating migration into the airpouch with either MSU or IL-1β led to a significant reduction of neutrophils in the airpouch (**Figure 27B**).

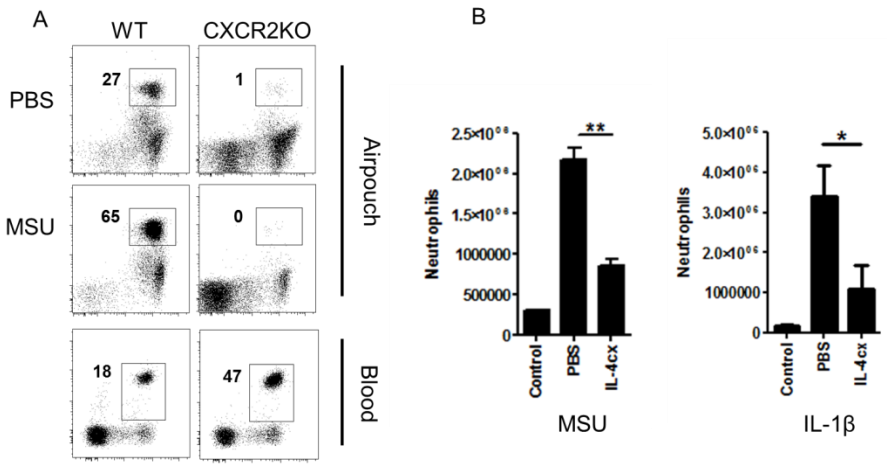

Figure 27. Neutrophil migration in vivo is impaired by IL-4cx. (**A**) Flow cytometric analysis of flushed airpouch content after overnight treatment with MSU crystals in wild-type B6 and CXCR2$^{-/-}$ (CXCR2KO) mice. Lower panel shows blood neutrophil compartment in wild-type and CXCR2$^{-/-}$ mice. (**B**) Wild-type B6 mice harboring an airpouch were treated intravenously with either PBS or IL-4cx, followed 15 minutes later by PBS (control), MSU or IL-1β into the airpouch. Shown are total migrated neutrophils. Data are representative of one out of 2-3 independent experiments with a total of at least 5 animals per condition. *P<0.05; **P<0.01.

8 Downstream signaling in neutrophils upon IL-4 stimulation

8.1 IL-4 induces STAT6 phosphorylation in neutrophils

As stated above, IL-4 is able to induce STAT6 phosphorylation upon signaling. We therefore tested whether IL-4 was also able to phosphorylate STAT6 in CD11b^{+} Ly6G^{+} neutrophils. Isolated splenocytes were, after a short resting period, stimulated for 15 minutes with 50 ng/ml IL-4 and subsequently stained for pSTAT6 along with CD11b and Ly6G, showing an increase in phosphorylated STAT6 in CD11b^{+} Ly6G^{+} neutrophils (**Figure 28A**). Increased STAT6 phosphorylation was not observed in neutrophils from CD124$^{-/-}$ mice (lacking type I and type II IL-4Rs) and was only partially reduced in CD132$^{-/-}$ neutrophils (lacking only type I IL-4Rs), indicating that type I IL-4Rs were not crucial for this effect (**Figure 28B**). We also compared the ability of IL-4 and IL-13, both of which can use the type II IL-4R, to induce STAT6 phosphorylation on neutrophils in varying concentrations. Consistent with earlier findings [73], we were able to

show, that higher concentrations of IL-13 are needed to induce the same level of STAT6 phosphorylation (**Figure 28C**).

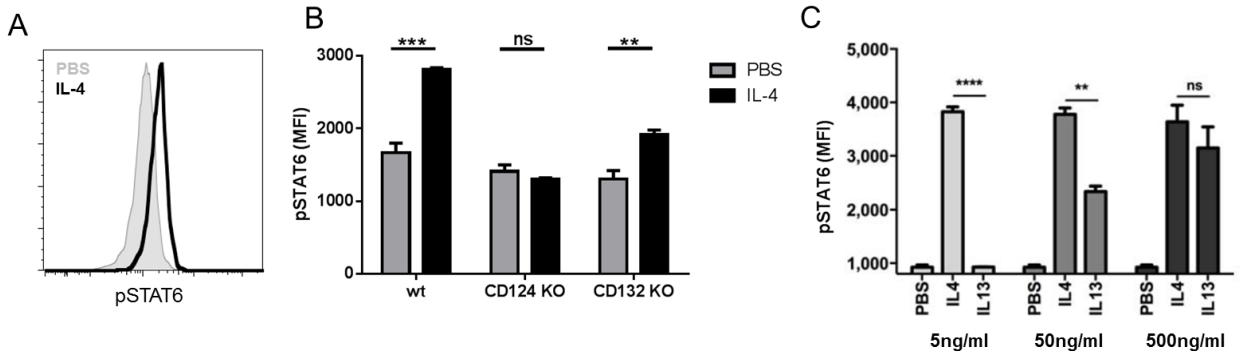

Figure 28. IL-4-induced STAT6 phosphorylation in neutrophils via signaling through type II IL-4R. (**A**) Histogram showing phosphorylated STAT6 (pSTAT6) gated on $CD11b^+$ $Ly6G^+$ neutrophils after resting WT B6 splenocytes were restimulated in vitro with PBS or 50 ng/ml IL-4. (**B**) Stimulation of splenocytes from wild-type, CD124-deficient or CD132-deficient mice with PBS or IL-4, followed by quantification of pSTAT6 in $CD11b^+$ $Ly6G^+$ neutrophils. (**C**) Quantification of pSTAT6 in $CD11b^+$ $Ly6G^+$ neutrophils following stimulation of wild-type splenocytes with increasing amounts of IL-4 or IL-13. Data are representative of 2-3 independent experiments with 2 animals per condition. *P<0.05; **P<0.01; ****P<0.0001; ns = not significant.

8.2 IL-4 induces p38 MAPK phosphorylation in neutrophils

We next sought to investigate downstream molecules of the IL-4R on neutrophils. To this end, we focused on the phosphatidylinositol 3-kinase (PI3K) and p38 mitogen-activation protein kinase (MAPK) pathways, as previous work has shown neutrophil chemotaxis towards CXCR2-binding chemokines to depend on PI3K, whereas activation of p38 MAPK by end target chemoattractants, is able to suppress PI3K-mediated migration of neutrophils [21, 47].

As expected [47, 101], chemotaxis of murine neutrophils towards CXCR2-binding chemokines was p38-independent, but relied on PI3K, as demonstrated by using the p38 MAPK inhibitor SB203580 and the PI3K-blocking agent LY294002 (**Figure 29A**). Most notably, in neutrophils migrating towards CXCL1 in vitro, concomitant use of the p38 blocker SB203580 reconstituted

the chemotactic capacity of IL-4-treated neutrophils to levels comparable with controls (**Figure 29B**). These data suggested that IL-4cx affected p38 MAPK signaling.

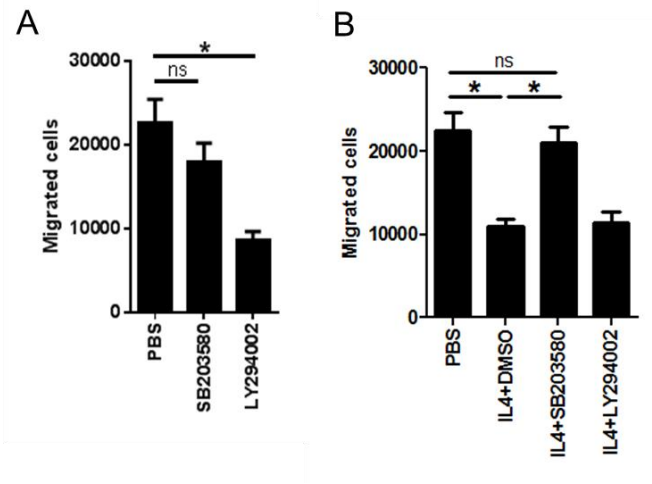

Figure 29. IL-4-mediated migration inhibition requires p38 MAPK. (**A**) CXCL1-induced migration of purified murine neutrophils was analyzed upon treatment in vitro with PBS, p38 inhibitor SB203580, or PI3K inhibitor LY294002 and (**B**) PBS, IL-4 plus DMSO, IL-4 plus SB203580, or IL-4 plus LY294002. Shown are total migrated cells. Data are representative of at least 2 independent experiments with 2 mice per condition. *P<0.05; ns = not significant.

In line with other publications [77], we showed that IL-4 is able to induce p38 phosphorylation in neutrophils in vitro already within 5 minutes (**Figure 30A**). p38 phosphorylation could be blocked by using the selective p38 inhibitor SB203580 in vitro (**Figure 30B**) and in vivo (**Figure 30C**) [102]. Administration of the p38 blocker SB203580 to animals receiving G-CSFcx plus IL-4cx corrected the in vivo deficiency of neutrophils induced by IL-4cx, thus resulting in neutrophil percentages and counts in the blood of these animals that were comparable to G-CSFcx treated mice (**Figure 30D** and **30E**).

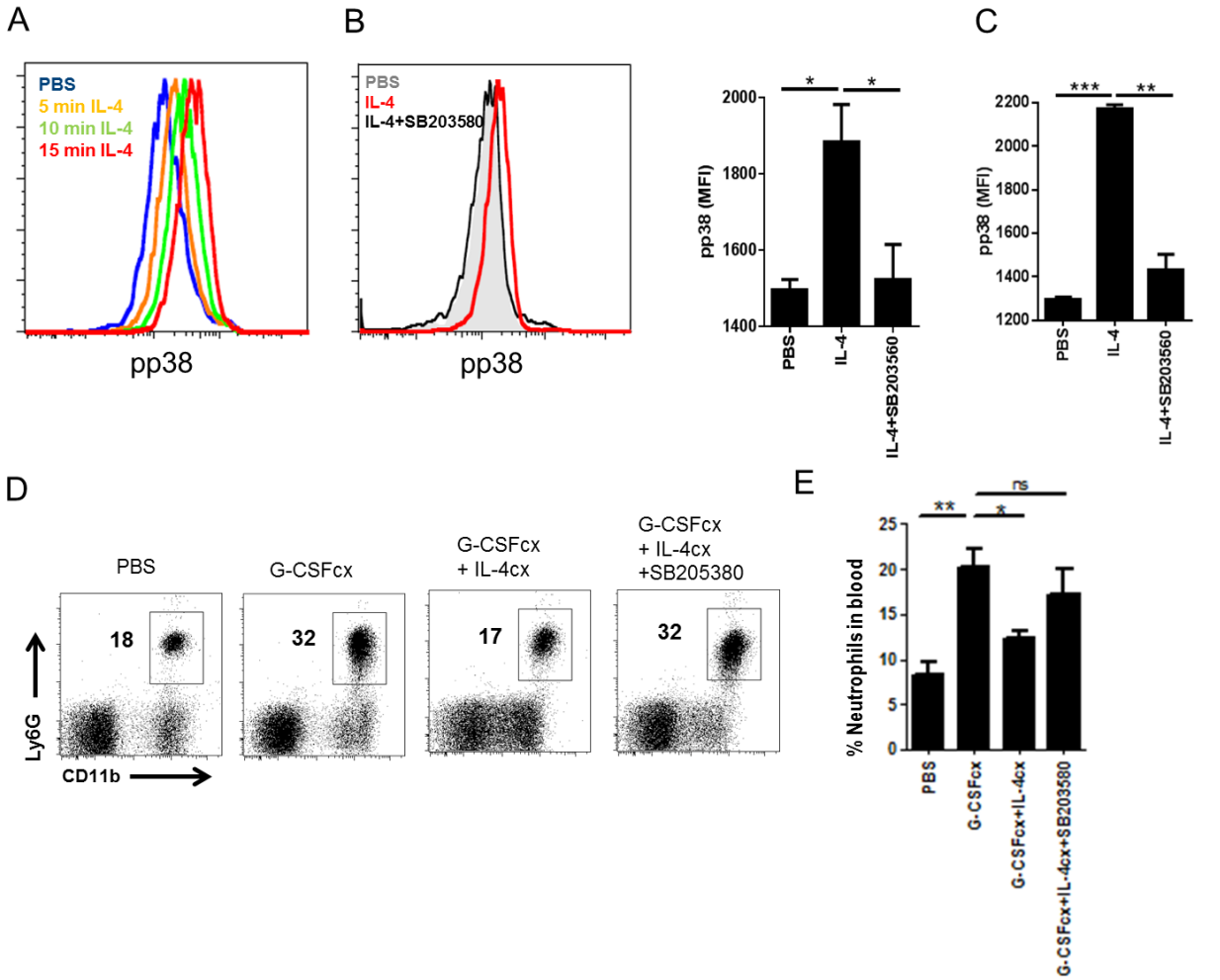

Figure 30. IL-4-induced p38α MAPK phosphorylation antagonizes G-CSF in mobilizing neutrophils to the blood. (**A**) Histograms show IL-4-induced p38 phosphorylation in $CD3^-$ $CD11b^+$ $Ly6G^+$ neutrophils after 5 minutes (orange line), 10 minutes (green line) or 15 minutes (red line) of stimulation of splenocytes with 30 ng/ml IL-4 or PBS (blue line). (**B**) p38 phosphorylation in $CD3^-$ $CD11b^+$ $Ly6G^+$ neutrophils after (**B**) in vitro or (**C**) in vivo treatment with PBS, IL-4 plus DMSO, or IL-4 plus p38 inhibitor SB203580, showing histograms (**B**, left graph) and MFI values of p38 phosphorylation (**B**, right graph, and **C**). (**D-E**) B6 wild-type mice were treated with PBS, G-CSFcx, G-CSFcx plus IL-4cx, or G-CSFcx plus IL-4cx plus SB203580 for 3 days. Shown are (**D**) dot plots and (**E**) frequencies of $CD3^-$ $CD11b^+$ $Ly6G^+$ neutrophils from blood 16 hours after the last injection. Data are representative of 2-3 independent experiments with 2-5 mice per condition. **P*<0.05; ***P*<0.01; ****P*<0.001; ns = not significant.

9 Diminished ROS production in neutrophils upon IL-4cx stimulation

The above-mentioned data demonstrate that IL-4 directly binds to neutrophils to inhibit their migration as well as their expansion and recruitment from the bone marrow into the circulation. Additionally, IL-4 might impact effector functions of neutrophils, including the generation of ROS to kill ingested bacteria [103, 104]. Thus, we measured ROS activity in $CD11b^+$ $Ly6G^+$ neutrophils by assessing the oxidation of dihydrorhodamine to rhodamine, following treatment of mice with PBS, IL-4cx, G-CSFcx, or G-CSFcx plus IL-4cx. Strikingly, ROS production was markedly decreased in neutrophils of mice given IL-4cx (**Figure 31A**) or CSFcx plus IL-4cx (**Figure 31B**) in comparison to PBS or G-CSFcx-treated animals. Even though G-CSFcx-treated mice seemed to have a lower percentage of ROS^+ cells, the absolute count of ROS^+ cells was significantly increased. The same pattern of diminished ROS production was also found in mice infected previously with 10^5 cfu LM (**Figure 31C** and **31D**). This reduction could result from an egress of not yet fully matured neutrophils from the bone marrow or by the fact that G-CSF administration leads to a certain release of ROS.

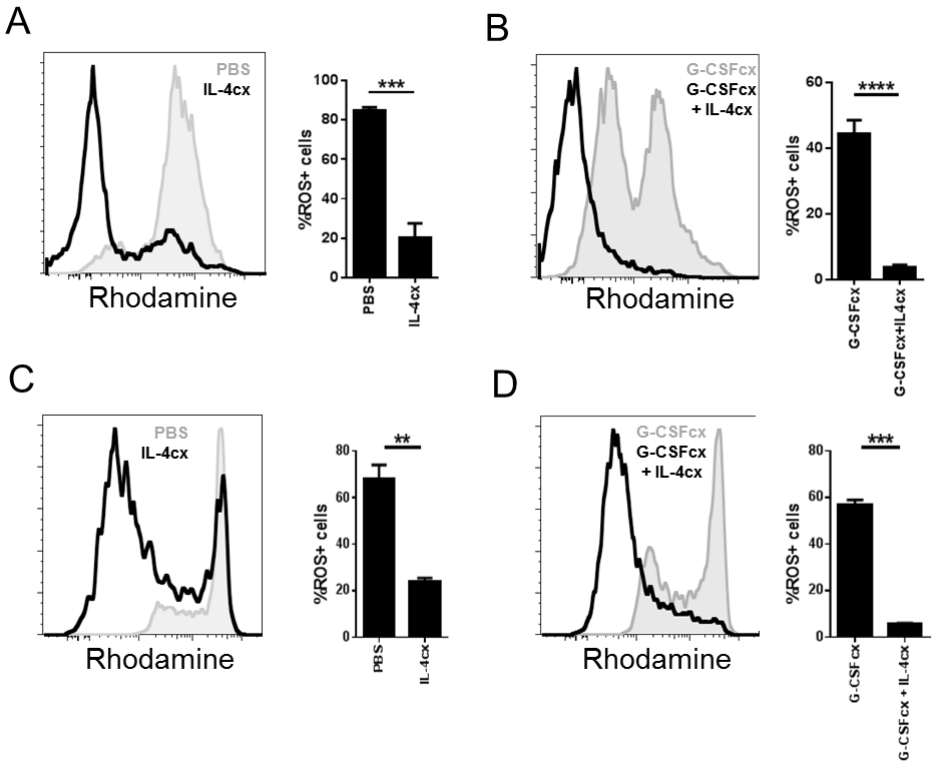

Figure 31. **Neutrophil effector functions are hampered by IL-4cx.** (**A-B**) B6 wild-type mice were treated with (**A**) PBS or IL-4cx or (**B**) G-CSFcx or G-CSFcx plus IL-4cx for 3 consecutive days, and 16 hours after the last injection ROS production was assessed by measuring oxidation of 1,2,3-dihydrorhodamine to rhodamine. Histograms show rhodamine$^+$ cells in CD3$^-$ CD11b$^+$ Ly6G$^+$ neutrophils and graphs summarize percentages of rhodamine$^+$ neutrophils in indicated animals. (**C-D**) Mice were pretreated as in A and infected i.v. 16 hours after the last cytokine treatment with 10^5 cfu Listeria monocytogenes. ROS production was assessed 24 hours post infection. Data are representative of 2-3 independent experiments with at least 3 mice per condition. ***P<0.001; ****P<0.0001; ns = not significant.

10 Neutrophils in cutaneous infection

10.1 IL-4cx dampens neutrophil influx into the skin

To investigate the role of IL-4 in the recruitment of neutrophils to the skin and muscles, a mouse model of necrotizing fasciitis was used [94, 95]. Group A Streptococcus M1T1 5448 strain (GAS M1) is an invasive clinical isolate derived from a patient with streptococcal toxic shock syndrome/necrotizing fasciitis (STSS/NF) [105]. As neutrophils are known to infiltrate rapidly upon cutaneous infections [106] we assessed their influx 6 hours post infection. Mice,

pretreated with IL-4cx for 3 days prior to infection, showed a marked decrease in infiltrating neutrophils, whereas neutralization of endogenous IL-4 by injection of anti-IL-4 mAb (11B11) 1 day prior to and on the day of infection led to strongly enhanced infiltration of neutrophils (**Figure 32A** and **32B**). In line with our previous data, mice pretreated with IL-4cx were not able to expand their neutrophil counts upon infection as shown by decreased cell numbers in blood (**Figure 32C**).

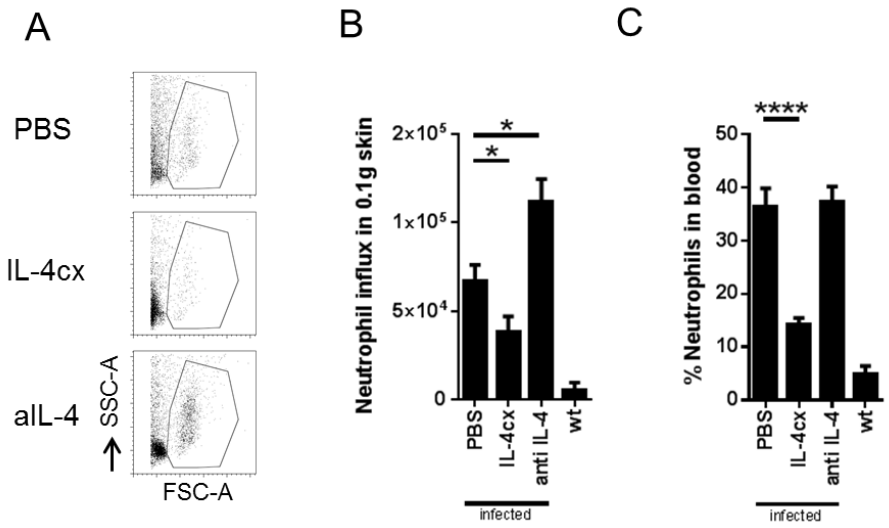

Figure 32. Exogenous and endogenous IL-4 inhibits neutrophil accumulation in infected skin. $3*10^7$ cfu of GAS M1 was injected subcutaneously into the shaved flank of wild-type B6 mice pretreated with PBS or IL-4cx or continuously treated with anti-IL-4. Infiltrating cells were analyzed 6 hours after infection by flow cytometry. (**A**) SSC-A and FSC-A plotted total skin. Shown is quantification of $CD11b^+$ $Ly6G^+$ neutrophils in (**B**) skin and (**C**) blood. Data are pooled from 2 independent experiments with a total of 8 mice per condition. *<0.05; ****P<0.0001.

10.2 Neutralizing endogenous IL-4 leads to clinical benefit

To demonstrate that altered neutrophil influx upon IL-4cx or anti-IL-4 treatment has a clinical effect on skin infection we determined bacterial burden in the skin 72 hours post infection. Indeed, mice pretreated with IL-4cx showed 4 times more bacteria present in the skin, whereas mice depleted of their endogenous IL-4 had a tendency towards lower cfu (**Figure 33A**). Enhanced IL-4 levels led to increased lesion size after 48 hours which, however, was not evident after 72 hours. This is most likely due to normalization of IL-4 levels after 72 hours as IL-4cx

treatment was discontinued one day before infection (**Figure 33B**). By measuring the lesion size it became even more evident that neutralizing IL-4 was beneficial as the lesion size was only about half at 72 hours after infection compared to untreated mice (**Figure 33C**).

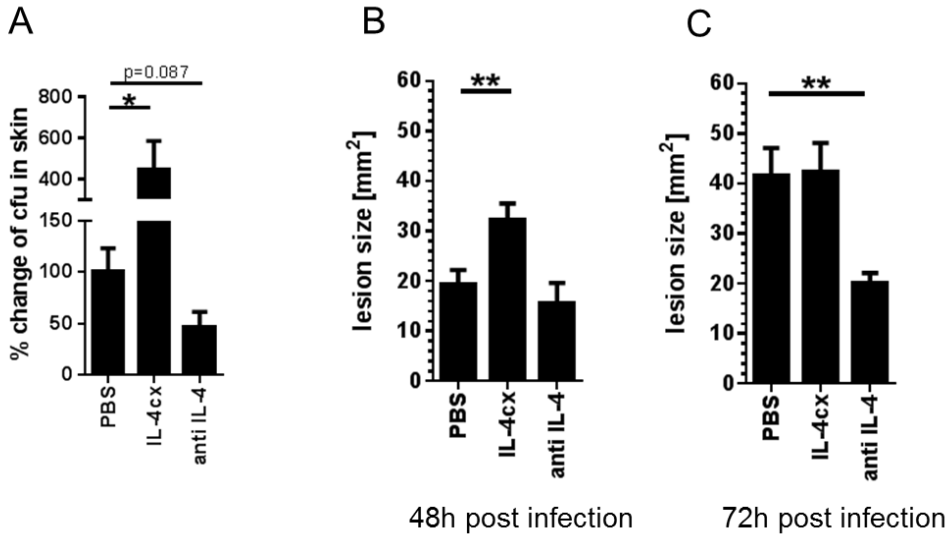

Figure 33. IL-4 leads to increased bacterial burden and increased size of skin lesion. $3*10^7$ cfu of GAS M1 was injected subcutaneously into the shaved flank of wild-type B6 mice pretreated with PBS or IL-4cx or continuously treated with anti-IL-4. (**A**) Cfu in skin was analyzed 72 hours post infection and normalized to PBS treated mice. Skin lesion was determined (**B**) 48 hours and (**C**) 72 hours post infection. Data are pooled from 2-3 independent experiments with a total of 8-11 mice per condition. *<0.05; **P<0.01.

VI DISCUSSION

Acute inflammation following tissue damage or infection is usually mediated by the release of a series of chemokines and cytokines by stromal and immune cells, including IL-1β, TNF-α, G-CSF, and GM-CSF. Many of these cytokines, most notably G-CSF, mobilize neutrophils from the bone marrow to the circulation wherefrom neutrophils are guided to the inflamed tissue by chemokines and end-target chemoattractants [13, 103, 104, 107]. Once the insulting stimulus is removed, neutrophils are eliminated both by a passive process via their short life-span and by factors inhibiting further neutrophil recruitment and activation [33].

In this thesis we investigated the action of IL-4, in the form of IL-4cx, on neutrophil expansion and recruitment to the periphery following G-CSF production upon systemic Listeria monocytogenes or cutaneous group A Streptococcus infection. Alternatively, we used G-CSFcx administration to mimic a systemic infection. We found that IL-4cx signals potently antagonized neutrophil recruitment both following systemic administration of G-CSFcx and bacterial infection. Conversely, several studies indicated a synergistic effect on neutrophils of IL-4 co-administered with G-CSF leading to a marked increase in neutrophil numbers [108, 109]. In a preliminary experiment (data not shown) we found the same effect after 24 hours, which however was reversed after continuous administration of IL-4cx, suggesting initially a stimulatory effect of IL-4cx on neutrophils which then is reversed. One explanation, which however needs further investigation, could be an altered response of neutrophils towards IL-4, for example by up- or downregulation of one of the IL-4R subtypes, after the cells were stimulated by G-CSF. Our data showed, consistent with others [80], increased maturation rate of neutrophils stimulated with G-CSF and IL-4 but not IL-4 alone, supporting the hypothesis of an altered IL-4 responsiveness in G-CSF stimulated neutrophils.

As shown in mixed bone marrow chimeras of wildtype and CD124-deficient cells, IL-4cx stimulated neutrophils directly, indicating the presence of IL-4R on neutrophils. It was suggested that type I IL-4R are expressed on hematopoietic cells whereas type II IL-4R are found on

stromal cells [73]. No effect of IL-4cx was seen when both receptor subtypes were missing; conversely, CD132-deficient neutrophils behaved comparable to wild-type neutrophils when exposed to IL-4cx in vivo, suggesting that IL-4 signaled through type II IL-4Rs to mediate these effects. Type II IL-4R, but not type I IL-4R, signaling leads to activation of STAT3 [73], STAT3 signaling activates SOCS3, which is a negative regulator of G-CSF signaling in neutrophils [32].

The literature concerning neutrophil survival after IL-4 stimulation is somewhat inconsistent, showing either decreased apoptosis rate [77, 79] or, in line with our data, no effect on neutrophil survival [110]. However, upon co-stimulation of G-CSF and IL-4 the apoptosis rate of bone marrow neutrophils was increased after 24 hours, suggesting a changed responsive rate of G-CSF stimulated neutrophils towards IL-4. The enhanced responsiveness towards IL-4 after G-CSF stimulation of neutrophils can be explained by the fact, that G-CSF led to an increased type II IL-4R expression, whereas the expression level of the type I IL-4R was not affected. This finding is consisting with the in vivo data showing no crucial need for CD132, one of the receptor subunits of the Type I IL-4R. It was previously shown that CD124 gets upregulated on neutrophils during acute inflammation; however the molecule mediating this effect could not be determined [81]. As acute inflammation leads as well to enhanced secretion of G-CSF [111], it is likely that this cytokine mediated the enhanced IL-4R expression.

Signaling of IL-4 led not only to increased retention of neutrophils in the bone marrow by upregulation of CXCR4, but as well to decreased sensitivity towards CXCR2-binding chemokines by downregulation of the latter receptor. IL-4 further led to a p38 MAPK dependent inhibition of neutrophil migration towards CXCR2-binding chemokines in vitro and in vivo. The literature regarding the role of IL-4 in neutrophil chemoattraction is controversial, some data suggesting stimulation others inhibition of neutrophil migration.

Enhanced chemotaxis of neutrophils due to IL-4 was demonstrated to depend on increased secretion of CXCL8 –the human functional analogue of CXCL1 and CXCL2 in mice- either way directly from endothelial cells or indirectly through activation of the dual oxidases [112-114].

In a model of α-galactosylceramide-induced hepatitis ablation of IL-4, the IL-4R, or its downstream signaling molecule STAT6 ameliorated neutrophil infiltration [115]. Other studies showed that IL-4 inhibited granulocyte infiltration and phagocytic activity in methylated BSA induced pleuritis in mice and mycobacteria-induced arthritis in rats [116, 117]. Our data and results from others showed further, that systemic administration of IL-4 antagonizes IL-1β or MSU crystals in attracting neutrophils to an air-pouch [84]. Moreover, mice deficient in both IL-4 and IL-13 demonstrated increased neutrophil infiltration into the liver following Schistosoma japonicum infection [118]. Further it was postulated that endogenous IL-4 produced during glomerulonephritis induced by heterologous anti-glomerular basement membrane Abs suppressed neutrophil influx and limited thereby tissue damage [83]. Summarized, more evidences point towards an inhibitory role of IL-4 in neutrophil chemotaxis.

It was suggested that IL-4 can as well alter neutrophil function, as IL-4 was able to suppress cytokine-mediated neutrophil activation, a critical step in exerting effector function afterwards [116]. We assessed the ability of ROS production of neutrophils ex vivo after IL-4cx treatment and could indeed observe a significantly decreased ability to reduce dihydrorhodamine to rhodamine after in vitro restimulation. In an in vitro assay on the other hand IL-4 boosted the ability of neutrophils to kill and phagocytose opsonized bacteria and, following stimulation with fMLP, to generate respiratory burst [80, 119].

Our data suggests a strong impact of the Th2 cytokine IL-4 in neutrophil homeostasis, migration und effector functions. This is of great interest as Th2 mediated diseases, including several allergic conditions, increase significantly in prevalence over the last decades [120]. Atopic dermatitis patients for example, show normal neutrophil counts in blood but diminished recruitment of neutrophils to the skin [121]. Up to 80–100% of these patients are colonized with Staphylococcus aureus even on nonlesional skin as compared to 5–30% of healthy controls [92]. This increased colonialization and neutropenia in the skin is even more surprising as Staphylococcus aureus is known to efficiently induce the recruitment of neutrophils [122]. Not only reduced neutrophil numbers were found in the skin, but as well their ability of

phagocytosis and ROS production was impaired [92]. Importantly, IL-4 was shown to be overexpressed in the skin of atopic dermatitis [121]. Our data generated in the model of cutaneous Streptococcus infection after IL-4cx treatment strongly supported this finding, showing significantly decreased levels of neutrophils in the affected skin. By neutralizing IL-4 during skin infection, we could prove that endogenous produced IL-4 during infection is as well of relevance for neutrophil infiltration and clinical outcome during bacterial colonization of the skin.

Our data therefore raises the question if therapeutic strategies targeting IL-4 – IL-4R interaction or p38 MAPK signaling could restore the innate immune response in patients suffering from atopic dermatitis or other allergic diseases. Furthermore, it could give another explanation why IL-4 treatment seems to be protective in models of rheumatoid arthritis [123, 124], as it was shown that neutrophils are as well an important disease causing mediator [125].

Taken together our data strongly suggest a clinical implication of direct binding of IL-4 to neutrophils and thereby inhibiting their responses during bacterial infections. This finding could even find relevance in non-infectious inflammations like rheumatoid arthritis or the systemic inflammatory response syndrome, which is characterized by the massive recruitment of immature neutrophils [126].

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VIII ACKNOWLEDGMENTS

I would like to express my special thanks to my advisor Professor Dr. Onur Boyman, you were not only an extraordinary supervisor for me and my research but as well exceptionally encouraging and motivating in all the ups and downs during the past four years. I could not have imagined having a better mentor for my PhD.

Further I would also like to thank my committee members Professor Dr. Cornelia Halin Winter and Professor Dr. Burkhard Becher for their excellent comments thereby helping to advance my project and the nice and productive atmosphere we had during our meetings. I really appreciate that I could contact you at any time and you were helping me out with my questions.

A special thanks goes as well to all the past and present members of our laboratory. You are not only colleagues for me who helped me out with scientific questions and problems and supported me when I needed assistance, but as well became true friends.

Further I would like to take this opportunity to express gratitude to Professor Dr. Annelies Zinkernagel and Nadia Keller who introduced me to the field of cutaneous infections. I enjoyed the stimulating collaboration and scientific discussion with you and highly appreciate all the effort you put into this project.

Last but not least I would like to thank my friends and family. Words cannot express how grateful I am for all the support I got from my mother, my father, my brothers including my sister-in-law, my two gorgeous nieces and my beloved boyfriend Patrick. You were more than understanding when I had spontaneously to cancel my appointments or had to work through the weekends.

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Forschungskredit Candoc of University Zurich

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